

BEHAVIOR/STRESS INTERACTIONS
IN DIABETES MELLITUS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1986

ACKNOWLEDGEMENTS

I decided early in my academic training to pursue a very multidisciplinary course. This has not always been an easy approach. I feel extremely fortunate to have found a program such as the one at the University of Florida, the Center for Neurobiological Sciences, which has the specific goal of bringing together people from different fields of study. My committee reflects this atmosphere of sharing, and it has been a great joy to feel welcome in the laboratories of neuroscientists and physiologists, as well as those of psychologists.

To my committee members, Drs. Adrian Dunn, Melvin Fregly, William Luttge, Merle Meyer and Carol VanHartesveldt, I wish to express thanks for so many helpful interactions as I planned and executed my dissertation work. Special gratitude must be extended to my advisor, Neil Rowland, who in addition to the usual difficult tasks involved in training a new scientist, was called upon for assistance in both professional and personal crises.

In addition to this wonderful professional family, I owe a great deal to some loved ones. First, thanks are extended to my children, Pam and Greg Bellush, who haven't had very much of a mother for several years, but who have been

unfailingly supportive and proud of my endeavors. Thanks are also extended to Dr. Bill Henley, my very best friend, my partner. I'm quite certain that without his constant optimism and encouragement and his unflagging faith in me, this dissertation might not have become a reality.

Thanks must go, finally, to my parents, Dorothy and John Jose, who supported me in ways too numerous to count throughout my life, and in particular during my difficult years in college. They have both died during my years in school and therefore cannot be here to see the culmination of all the years of struggle. This saddens me deeply, for they so deserved to share in the rewards. This dissertation is my tribute to their memory.

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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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December 1986

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The responses of diabetic and nondiabetic rats to two stressors were investigated. In the first series of experiments, retention of passive avoidance was examined. Possible state-dependent effects of glycemic state were evaluated in separate diabetic and nondiabetic groups. Additionally, retention of diabetic and nondiabetic rats was compared. Also compared were corticosterone concentrations, catecholamine excretion and catecholamine concentrations in brain. Changes in glycemic state produced no state-dependent effects in retention. However, diabetic rats showed better retention for the task than nondiabetic rats. Associated with retention differences, diabetics had higher epinephrine excretion and nondiabetics lower excretion after

footshock training, relative to baseline measures. Diabetics had higher norepinephrine excretion than nondiabetics at both times. Diabetic rats had higher norepinephrine concentrations in amygdala, hypothalamus and brainstem, and higher dopamine concentrations in hypothalamus. These data suggest that in diabetes mellitus, alterations in peripheral and central catecholamine functioning may result in greater responsivity to stressful episodes. Another experiment evaluated metabolic parameters in insulin- and saline-treated diabetic and nondiabetic rats after 72 hours in a cold environment. Corticosterone concentrations were significantly elevated after three days of cold, and insulin had opposite effects upon this parameter in diabetic and nondiabetic rats. Diabetics had higher norepinephrine excretion than nondiabetics during the first 24 hours of cold and lower dopamine excretion during the last 24 hours. In the cold, dopamine in frontal cortex was influenced in opposite ways by insulin treatment, with concentrations in diabetics increased and those in nondiabetics decreased. Diabetic rats had higher norepinephrine concentrations in frontal cortex and brainstem irrespective of insulin treatment. These biochemical alterations in diabetic rats, and the failure of insulin to normalize them, suggest that both acute and chronic stresses may have different effects upon the diabetic, because of different biochemical status

produced by diabetes and the failure of insulin to normalize fully the physiological processes involved in adaptation to stress.

CHAPTER I DIABETES, STRESS AND BEHAVIOR

Clinical Perspectives

Psychosocial Aspects of Diabetes

The streptozotocin-diabetic rat has provided a useful model for the investigation of treatment, prevention, causal factors and complications of insulin-dependent diabetes mellitus (Mordes & Rossini, 1981). However, very little of the animal research has focused upon questions related to behavioral interactions with metabolic control in diabetes. This is somewhat surprising, given the vast clinical literature pertaining to psychological aspects of this endocrine disorder in humans.

Menninger (1935), Dunbar, Wolfe and Rioch (1936) and Daniels (1939) published some of the earliest formulations concerning psychological aspects of diabetes. All of these theorists emphasized the contribution of both personality factors and environmental stresses in the precipitation of diabetes, as well as in the course of the disorder. In addition, they all leaned heavily on psychoanalytic concepts such as that of unconscious conflict which could lead to physiologic expression. Thus, Daniels (1939) noted that certain personality "types" were predisposed to respond to

anxiety-provoking events by becoming clinically glycosuric and hyperglycemic as a consequence of autonomic activation. The environmental stressor most often linked to the onset of diabetes was the loss of a loved one or object. The psychoanalytic interpretation of the ensuing events involved a mixture of hostility at the perceived abandonment and sadness at the loss. These emotions, if not expressed in other ways, could result in autonomic events ultimately producing diabetes (Daniels, 1939).

Menninger (1935) and Dunbar et al. (1936) stated similar theories relating personality factors and environmental stresses to the onset and control of diabetes. Menninger (1935) noted an increased incidence of glycosuria among patients with other psychosomatic illnesses, particularly depression.

Numerous studies have been published in the intervening years from 1935 to the present which have attempted to demonstrate either environmental stresses or personality factors involved in the predisposition to diabetes. For example, Stein and Charles (1971) suggested that a large number of diabetics experienced the death of a loved one prior to the onset of the disorder. However, these authors included deaths occurring as long as several years prior to the development of diabetic symptomology. Hinkle and Wolf (1952) also argued that stress could be an important factor in diabetes onset. Stress continues to be studied as a

variable of importance, both in the precipitation of diabetes and in maintenance of metabolic stability (Johnson, 1980). Indeed, it has been suggested recently that diabetes represents a state of uncontrolled stress (Zaidise & Bessman, 1984).

As for predisposing personality factors, it has been difficult to determine whether personality traits such as dependence and anxiety cause, or are caused by, diabetes. A number of studies have reported indications of increased depression, anxiety or other adjustment problems among diabetics (e.g. Swift, Seidman, & Stein, 1967; Simonds, 1977; Sullivan, 1979; Murawski, Chazan, Balodimos, & Ryan, 1970; Sanders, Mills, Martin, & DeL. Horne, 1975). However, a number of methodologic flaws such as subjective interview techniques, invalid or unreliable tests (e.g. projective tests such as the Rorschach), questionable selection of both diabetic and control populations have marred the interpretation of many of the results. Moreover, other studies have found good adjustment, in general, among diabetics (Kovaks, Feinberg, Paulauskas, Finkelstein, Pollock, & Crouse-Novak, 1985). There is some evidence that a relationship exists between diabetes and depression (Lustman, Amado, & Wetzel, 1983) but the precise incidence and prevalence of depression in diabetics and the impact of depression upon blood glucose concentration have not been

established (Kymissis, Shatin, & Brown, 1979; Lilliker, 1980).

It has been questioned whether any consistent personality differences or psychological problems actually characterize diabetics as a group (Bruch, 1949; Johnson, 1980; Johnson & Rosenbloom, 1982). Nonetheless, there is a continuing concern that psychosocial adjustment is a relevant issue bearing upon metabolic status in the treatment of diabetes (Johnson, 1980). For example, the lifestyle of the diabetic is subject to numerous constraints. It is necessary to self-inject insulin once or several times each day, to test the urine or blood frequently for determination of glucose concentrations, to eat carefully specified types of foods at strictly specified times, and to avoid many of the most desirable foods altogether. No matter how carefully these restrictions are observed, episodes of insulin shock occur from time to time. In addition, these individuals live with the knowledge that they are at greater risk than other individuals for the development of nephropathy, retinopathy and neuropathy (Johnson, 1980).

While good glycemic control is likely the best way to avoid both short-term and long-term problems, compliance to the diabetic regimen continues to be a major problem in treatment (Johnson, 1980). This is not altogether surprising. Given the numerous details of diabetes

management listed above, it is clear that even under the best of circumstances such a demanding lifestyle could become emotionally taxing. Moreover, there are often no immediate consequences of the failure to comply with the restrictions. In fact, some anecdotal evidence suggests that the diabetic individual may actually feel more energetic when somewhat hyperglycemic than when normoglycemic (Bruch, 1949).

When levels of insulin in blood are inadequate, blood glucose rises, and ketoacidosis and diabetic coma may also occur in some diabetics. Overinsulinization, on the other hand, can produce unsettling symptoms such as sweating and dizziness, shock, coma or even convulsions, which can be both embarrassing and life-threatening. Given the tedious process involved in maintenance of appropriate glycemic levels, it is not difficult to understand why compliance problems often occur, particularly during difficult developmental stages such as adolescence (Johnson, 1980). Thus, rebellion against the environmental stringencies imposed by diabetes may result in metabolic derangements which may have both behavioral and physiological consequences.

There is evidence that stress may directly influence metabolic parameters even when the diabetic regimen is followed (Johnson, 1980). For patients referred to as "brittle diabetics," who are particularly susceptible to the

development of hyperglycemia and ketoacidosis (Tattersall, 1981), establishing metabolic stability can be a constant struggle. Even diabetics who are not particularly brittle, and who have not omitted scheduled insulin doses, may experience sudden ketoacidosis. In one report of 25 such individuals (MacGillivray, Bruck, & Voorhess, 1981), stressful events, including infection and psychological stress, could be identified in 20 cases to have occurred prior to the onset of the metabolic crisis. It was postulated that stress overwhelmed the control imposed by the prescribed dose of insulin, leading to hyperglycemia, ketoacidosis and striking elevations of the stress-related hormones, cortisol and epinephrine.

Much attention has been given both to compliance problems and to the stresses inherent in family and individual adjustment to diabetes, as they relate to metabolic stability. It is clear that disturbed family interactions are associated with disruptions in diabetic control (Bruch, 1949; Johnson, 1980). However, much less consideration has been given in the clinical literature to the possibility that primary alterations in metabolism in diabetic individuals may actually lead to behavioral changes (Johnson, 1980). These metabolically induced behavioral alterations could, in turn, cause further disruption in psychosocial interactions, as well as in metabolic stability. It is conceivable that hormonal or metabolic

changes in the diabetic individual may interfere with comprehension of the instructions given by the physician. Thus, compliance problems may not be due solely to rebellion against an austere lifestyle. Altered metabolism may also contribute to behavioral problems such as depression and anxiety (Leigh & Kramer, 1984).

Numerous investigators have attempted to determine whether there are behavior problems which arise specifically in connection with diabetes (Bruch, 1949). This is, of course, another version of the hypothesis of a diabetic personality, with personality here seen as the effect rather than the cause of diabetes. Just as no constellation of personality traits seems to lead to onset of diabetes, no such constellation can be identified consistently in established diabetes (Dunn & Turtle, 1981; Johnson, 1980; Bruch, 1949). Nonetheless, it may be that at least some diabetics are vulnerable to depression or anxiety (Lustman et al., 1983), and perhaps all diabetics may be more sensitive to stress than are nondiabetics (Johnson, 1980). Many studies reporting no difference in the psychosocial adjustment of diabetics relative to that of nondiabetics (e.g. Kovacs et al., 1985) have been careful to establish "representative" samples of diabetics and to test subjects under carefully controlled conditions. It is possible that specific groups of diabetics, for example "brittle diabetics" or diabetics with early onset diabetes are

uniquely at greater risk than other diabetics for development of behavioral problems. Little investigation of such high risk diabetics has been done, yet differences may be found by comparing various subgroups of diabetic individuals (Johnson, 1980), or by intentionally manipulating the stress of the testing situation (Kemmer, Bisping, Steingruber, Baar, Hardtmann, Schlaghecke, & Berger, 1986).

In one recent study (Linn, Linn, Skyler, & Jensen, 1983), Type I (insulin-dependent) diabetic men reported a larger number of stressful life events on the Holmes and Rahe Social Readjustment Scale than Type II (non-insulin dependent) diabetics. The Type I diabetics also perceived more stress in each episode, experienced greater anxiety, and suffered poorer immune function and metabolic control. Neither family support nor compliance differed between the two groups.

In another report (Jacobson, Rand, & Hauser, 1985) loss of glycemic control was related to the reported incidence of negative life events in diabetics experiencing early signs of retinopathy. Diabetics with no retinopathy and diabetics with long-standing retinopathy showed no such association between stress and metabolic stability. Thus, there was a relationship between glycemic control, psychological stress and metabolic deterioration during a specific extended period of time. Both this study and the study of Linn et

al. (1983) compared subgroups of diabetics. If all the diabetics had been evaluated together as a single group and compared with a control group of nondiabetic individuals, the important interactions between stress and metabolic stability may have been masked.

Finally, the majority of studies evaluating behavioral interactions with hormonal control in diabetes have emphasized the notion that the constraints of the diabetic lifestyle lead to behavioral problems which in turn lead to metabolic problems. It must also be considered that hormonal imbalances created by diabetes mellitus may not be totally normalized by insulin therapy, and are themselves the primary causes of behavioral problems. This latter possibility is a major impetus for using animal models of diabetes, in which compliance is not a problem, and metabolic state can be manipulated directly in order to determine the effects of metabolic parameters upon behavioral variables.

In summary, while there is not yet consensus as to the specific role played by psychological factors in metabolic stability in diabetic individuals, most current investigators do feel that there are behavior-hormone interactions that warrant further investigation (Johnson, 1980; Fisher, Delamater, Bertelson, & Kirkley, 1982).

Cognitive Changes in Diabetes

In addition to the psychosocial factors that may exert influence upon the course of diabetes, there may also be cognitive changes in diabetic individuals. A number of neuropsychological functions have been compared in diabetic and nondiabetic individuals, and some potentially important differences have been noted.

For example, electroencephalograms (EEGs) have been reported to be abnormal in many more diabetic than nondiabetic individuals (Eeg-Olofsson, 1977; Haumont, Dorchy, & Pelc, 1979). Such abnormalities in diabetic brainwave patterns include lower alpha frequency and increased paroxysmal activity. Abnormal paroxysmal activity has been correlated with behavioral and perceptual disturbances and with emotional lability in diabetic individuals and with the occurrence of severe hypoglycemic symptoms (Eeg-Olofsson, 1977). EEG abnormalities have also been shown to correlate significantly with poor diabetic control, as well as with retinopathy (Haumont et al., 1979).

Ack, Miller and Weil (1961) noted lower IQ scores on the Stanford-Binet test in diabetics with onset prior to age 5, when compared with IQ scores of nondiabetic siblings. No difference in IQ scores between diabetics with onset after age 5 and their nondiabetic siblings was found. A relationship was also suggested between the magnitude of IQ differences and the number of severe hyperglycemic and

hypoglycemic episodes experienced by the diabetic of early onset.

These findings were confirmed and extended in a more recent investigation by Ryan, Vega and Drash (1985) utilizing a comprehensive neuropsychological test battery which included the Wechsler Intelligence Scale for Children (WISC-R). Adolescents with diabetes onset prior to age 5 performed more poorly than later-onset diabetics and matched controls on virtually every test. These included measures of intelligence, school achievement, visuospatial ability, memory, motor speed and eye-hand coordination. Both age of onset and duration affected performance on tests. Rovet, Gore and Erlich (1983) obtained similar results when comparing diabetics with onset prior to age 3 to later-onset diabetics and nondiabetic siblings. Ryan et al. (1985) believe the deficits in early onset diabetics derive from the higher incidence of hypoglycemia leading to seizures and subsequent brain damage in these youngsters.

Decreased cognitive functioning has also been reported in older diabetics (Perlmutter, Hakami, Hodgson-Harrington, Ginsberg, Katz, Singer, & Nathan, 1984). These diabetics were type II diabetics (i.e. adult-onset diabetics). However, 56 were receiving daily insulin injections and 34 others required oral hypoglycemic agents. The major deficit found in these subjects when compared to nondiabetics was impaired memory retrieval. Performance was poorer in

diabetics with neuropathy or with elevated hemoglobin A_{1c}, an indicator of poor metabolic control of long duration.

Not all studies have reported deficits in diabetic cognitive performance. Fallstrom (1974) reported higher IQ scores on the WISC in diabetics than in nondiabetics. This was not the first study to obtain such results, but the previous reports of higher IQ scores in diabetics had been attributed to higher socioeconomic status of some samples of diabetics. However, Fallstrom's (1974) sample was balanced for socioeconomic status, and thus no simple confounding variable could be identified which explained her results. A relationship was found between age of onset of diabetes and IQ in boys but not girls, presumably because few girls in this sample had become diabetic before age 5.

In a study designed to evaluate the effects of maximal metabolic control versus standard insulin control in diabetics, 32 adults with duration of diabetes of about 20 years were administered, by telephone, the Wechsler Memory Scale. No differences in performance were found as a function of different treatment regimen. Thus, patients exposed to hypoglycemic episodes due to the rigid glycemic control of insulin pumps or multiple daily insulin injections appeared to suffer no memory impairments relative to diabetics receiving a single daily insulin injection. It should be noted, however, that all subjects tested here had had diabetes for 20 years and so may have suffered some

cognitive loss. No nondiabetic groups were included for comparison in this study. Despite these shortcomings, the study suggests that hypoglycemic episodes do not necessarily cause impaired cognitive function.

Flender and Lifshitz (1976) evaluated motor coordination, memory performance and concentration ability in diabetic and nondiabetic children at different blood glucose concentrations. Diabetics performed better than nondiabetic controls on the memory test in the hyperglycemic (150-300 mg/dl) and normoglycemic (91-150 mg/dl) ranges. One potential problem with this study was the unusual testing situation. Diabetics were tested hourly from 9 A.M. to 3 P.M., a rather long session. The nondiabetics were tested during glucose tolerance tests. Both of these conditions may have exerted unknown effects upon performance.

The effects of fluctuations in blood glucose concentration upon cognitive performance of diabetic college students were studied with the use of insulin infusions which maintained blood glucose concentration at 60 mg/dl, 110 mg/dl and 300 mg/dl in successive time periods (Holmes, Hayford, Gonzalez, & Weydert, 1983). Some impairment in performance was observed at both high and low blood glucose levels, with the greater impairment during hypoglycemia. It would have been of interest to see if nondiabetic individuals had suffered similar impairment at the lower

blood glucose levels, but no such subjects were studied. The results do address the issue of fluctuations in blood glucose experienced by diabetics routinely, and they suggest that such excursions in glucose concentration may have some cognitive impact.

Taken together, with the exception of Fallstrom (1974) and Flender and Lifshitz (1976), these studies generally agree that there are some cognitive deficits experienced by diabetic individuals. These deficits appear to be more prevalent in diabetics with early onset of the disease. Metabolic instability during neural development may thus be particularly damaging. The finding of alterations in EEG patterns in some diabetic individuals suggests that there may be physiological changes accompanying cognitive impairment. Another indication of organic damage is found in the lower flicker value of diabetics in the critical flicker fusion test (Ryan, Vega, Longstreet, & Drash, 1985). As with the issue of psychosocial adjustment of diabetic individuals, diabetic cognitive functioning will require further investigation to clarify the interaction between metabolic state and behavior. However, it seems clear that such interactions do occur in diabetes.

Experimental Perspectives

Hormonal and Neurochemical Correlates of Learning and Memory

In addition to the clinical findings which suggest a relationship between the metabolic changes and behavioral

alterations occurring in diabetes, there are experimental data which support the relevance of hormonal alterations in diabetes to learning and memory. In order to explore these experimental bases for investigating learning and memory in the streptozotocin diabetic rat, I will first summarize some of the known hormonal and neurochemical correlates of learning and memory. Then I shall describe some of the hormonal and neurochemical alterations in the streptozotocin diabetic rat, as well as in human diabetics.

State-dependent retention

One major experimental paradigm utilized to examine hormonal influences upon memory is that of state-dependent retention. In this paradigm, it has been demonstrated that if the internal state of subjects is different during training than it is during retention testing, then retention of the task may be attenuated relative to retention of the task if the internal state during retention matches the state existent during training (Overton, 1984). Training is often carried out in rats, and the task is often one-trial passive avoidance. Briefly, the animal is punished for making a response and thus learns not to make the response when given another opportunity to do so. The internal state of the animal can be altered by administration of drugs, including hormones. Izquierdo and Dias (1983) demonstrated state-dependent retention of passive avoidance in rats with exogenous administration of both ACTH and epinephrine.

Unfortunately, the notion of state dependency has become a standard explanation for any decrement in performance of a learned task, thus weakening its ability to contribute any clearcut explanatory power. Moreover, it has been difficult to defend the relevance of state dependent phenomena to real world situations. One such situation often cited is the loss of memory for events occurring during intoxication. However, state dependent retention is not necessarily the only, or even the best, explanation for such memory loss.

Diabetes mellitus, on the other hand, presents a situation in which there can be a great deal of fluctuation in glycemic state, and often in other metabolic parameters affected by glycemic state as well. Thus, diabetics may experience very high blood glucose which can be accompanied by elevated ketones, epinephrine and cortisol (MacGillivray et al., 1981). Alternatively, a number of diabetics also experience episodes of hypoglycemia, sometimes so severe that convulsions or coma ensue.

Such wide excursions in blood glucose concentration and the accompanying alterations in metabolic status may provide the basis for state-dependent retention. If so, performance in school or in any area of life could be affected in subtle ways. Experimental diabetes, induced in the laboratory, can provide the basis for determining whether state dependent retention occurs as a function of systematically altering metabolic state, and neurochemical correlates can be

ascertained as well. The clinical relevance of such studies is suggested in studies such as that by Holmes et al., (1983) in which altered glycemic state impaired cognitive performances. In addition, experimental diabetes provides a heuristic tool for the study of state dependent retention with more naturally occurring forms of alteration of internal state. Specifically, the loss of endogenous insulin and its replacement with exogenous hormone to restore normal blood glucose levels would allow modulation of numerous metabolic and neurochemical parameters which might underlie state dependency (Bartness & Rowland, 1983; Lozovsky, Saller, & Kopin, 1981).

Hormonal modulation of memory processing

In addition to providing an internal milieu which may serve as a contextual cue for remembering a training episode, hormones may actually influence the mechanisms involved in producing memory traces and in providing access to such memories. Peripheral hormones of two major axes have been implicated in learning and memory processes. First, ACTH and glucocorticoids, hormones of the hypothalamo-pituitary-adrenal (HPA) axis, have received a great deal of attention for their potential role in cognitive processes (McGaugh, 1983).

Hypophysectomy, which lowers plasma concentrations of both ACTH and glucocorticoids, impaired learning of an pole-jumping avoidance task (de Wied, 1964). Alternatively,

adrenalectomy, which elevates plasma levels of ACTH facilitated acquisition of a two-way active avoidance response, as did administration of ACTH. (Beatty, Beatty, Bowman, & Gilchrist, 1970). In all cases, administration of hormones to restore normal levels also restored performance to control levels.

Administration of ACTH to intact animals also influenced learning and memory. It delayed extinction of passive (Levine & Jones, 1965) and active (de Wied, 1971) avoidance responses and alley-running for food reward (Garrud, Gray, & de Wied, 1974). Administration of ACTH prior to training sessions led to an increase in the number of barpresses during the latter period of the training session, as well as during a subsequent extinction session (Guth, Levine, & Seward, 1971). ACTH also attenuated carbon dioxide-induced amnesia for a one-trial passive avoidance task (Rigter, van Riezen, & de Wied, 1974). The effects of ACTH were often noted when the hormone was administered immediately either after training (Gold & van Buskirk, 1976) or prior to retention testing (Rigter et al., 1974), suggesting that the effects of exogenously administered ACTH might be related to endogenous hormonal responses to training (Gold & Delanoy, 1981).

Thus, it was noted that following post-training administration of ACTH, there were both dose-dependent and time-dependent effects (Gold & Delanoy, 1981) upon

retention. Small doses enhanced, while larger doses impaired, memory for a one-trial passive avoidance task. Moreover, the effect of a given dose of ACTH interacted with the footshock intensity utilized in training, suggesting that hormone administration might be interacting with the endogenous hormonal response to footshock.

The effectiveness of post-training ACTH also depended upon administering the hormone within 10 minutes of the training trial. Administration of the hormone more than 10 minutes after training was without effect upon retention. This temporal gradient suggested the relationship of hormone administration to normally occurring post-training hormone release. Indeed, it was shown that footshock training produced large increases in plasma ACTH (van Wimersma Greidanus, Rees, Scott, Lowry, & de Wied, 1977). Moreover, these elevations peak 5 minutes after stress and return to baseline levels in 15 minutes. Interestingly, endogenous ACTH levels increased even more upon re-exposure to the fear cues associated with shock during retention testing than they did to footshock itself. It is thus possible that retention is to some degree related to the recurrence of elevated hormone concentrations.

Glucocorticoids have been shown to have their own effects upon learning, which are distinct from those of ACTH. For example, post-training corticosterone injections facilitated memory for a passive avoidance task in poorly

trained mice (Flood, Vidal, Bennett, Orme, Vasquez, & Jarvik, 1978). Cycloheximide, which reduces plasma corticosterone levels, attenuated memory for a passive avoidance task in rats when given 30 minutes prior to training (Cottrell & Nakajima, 1977). This attenuation could be prevented if corticosterone was also administered prior to training. Adrenalectomy facilitated extinction of alley-running for food reward, and corticosterone replacement normalized extinction (Micco, McEwen, & Shein, 1979).

The sympathoadrenal system has also been shown to influence memory processing. Depletion of peripheral norepinephrine (NE) impaired acquisition of a difficult (two-way) active avoidance task (Oei & King, 1980). Moreover, both epinephrine (EPI) and NE were elevated immediately following a 3mA 2-second shock, which produced good retention, but not following a 0.6mA shock, which produced poor retention (McCarty & Gold, 1981). Thus, post-training plasma levels of EPI and norepinephrine correlate with retention performance.

Post-training injections of EPI and NE enhanced retention for passive avoidance training (Gold & McGaugh, 1978), just as post-training ACTH did. It should be noted, however, that ACTH administration did not elevate plasma NE or EPI, nor did it affect the EPI response to footshock (McCarty & Gold, 1981). Thus, these two sets of plasma

hormone responses may be independent, or they may interact in the brain to modulate memory processing.

Glycemic state itself has been implicated in learning and memory. For example, hypoglycemic rats chronically treated with insulin (blood glucose=50 mg/dl) failed to learn an appetitive T-maze task (Clayson, 1971). Insulin-treated diabetic rats, which were essentially normoglycemic (blood glucose=119 mg/dl) learned the task more quickly than non-insulin-treated control animals. Unfortunately, no non-insulin-treated diabetic group was included in this study.

Gold (in press) found that post-training subcutaneous injection of 10 or 100 mg/kg of glucose enhanced retention of a passive avoidance task. Because the timecourse and dose-response of glucose modulation were similar to the modulation produced by EPI administration, Gold (in press) suggested that EPI-induced elevations of plasma glucose may be involved in normally occurring memory storage.

Neurochemical Correlates of Learning and Memory

The alterations noted in peripheral hormone and glucose concentrations following either training or testing episodes are likely related to concurrent events within the central nervous system related to the modulation of memory storage and retrieval (Gold & McGaugh, 1978). While the mechanisms underlying these processes are not yet fully understood, a number of findings suggest that they entail neurohumoral alterations.

For example, the turnover of dopamine in the central nucleus of the amygdala and the turnover of noradrenaline in the hippocampal dentate gyrus were elevated in rats showing good retention for passive avoidance (Kovacs, Versteeg, de Kloet, & Bohus, 1981). Turnover was measured by administering alpha-methyl-p-tyrosine and measuring disappearance of either dopamine or norepinephrine 2 or 4 hours after treatment.

Reductions in forebrain and brainstem norepinephrine of about 20%, measured 10 minutes after footshock training, were indicative of memory storage required to later perform a passive task (Gold & Murphy, 1980). Either larger or smaller depletions were accompanied by attenuated retention. Depletions of forebrain norepinephrine produced resistance to extinction as well (Mason, Roberts, & Fibiger, 1979).

Concentrations of 3,4-dihydroxyphenylacetic acid (DOPAC) have also been found to be affected by footshock training (Herman et al., 1982). While footshock itself was accompanied by elevations of DOPAC in frontal cortex, olfactory tubercle, nucleus accumbens and amygdaloid complex, returning the animals to the apparatus where they had been shocked led to elevation of DOPAC only in the anteromedial frontal cortex.

The hippocampus has also been implicated in learning and memory, and its role seems to be related to interactions with glucocorticoids (Micco et al., 1979). This system

seems particularly important in extinction, since hippocampectomy was shown to retard extinction of alley running for food, while adrenalectomy facilitated it. Systemic corticosterone administration following hippocampectomy did not restore extinction although hormone replacement did normalize extinction after adrenalectomy. Intrahippocampal hydrocortisone injection prevented amnesia for passive avoidance in rats produced by cycloheximide (Cottrell & Nakajima, 1977). Elevation of hippocampal serotonin 24 hours after footshock training was found to be associated with retrievability of the task (Ramaekers, Rigter, & Leonard, 1978).

Hormonal and Neurochemical Alterations in Diabetes

Both human and experimental diabetes are accompanied by a number of alterations in peripheral hormone concentrations. In the clinical literature, there are reports of alterations in both corticosteroid and catecholamine function. For example, diabetic individuals responded with a blunting of the normal suppression of cortisol when given dexamethasone (Cameron, Kronfol, Greden, & Carroll, 1984). In addition, plasma cortisol concentrations in diabetics have been reported to be higher than those of nondiabetics both in the early evening and in the early morning (Lebinger, Saenger, Fukushima, Kream, Wu, & Finkelstein, 1983). Asfeldt (1972) found normal day-night rhythms and baseline concentrations of cortisol in plasma of

diabetics. However, elevations in plasma cortisol concentration occurred in many diabetic individuals during hyperglycemic states without acidosis, as well as during perceived hypoglycemia, even when blood glucose concentration actually was normal. Thus, the excursions in blood glucose known to occur regularly in many diabetics (Holmes et al., 1983) could be accompanied by cortisol elevations.

When challenged with infusions of cortisol and epinephrine, diabetics experienced large increments in plasma glucose concentration, which were sustained for several hours (Shamoon, Hendler, & Sherwin, 1980). Controls showed no increments at all in plasma glucose concentration to cortisol infusion, and only transient increments to epinephrine infusion. Glucose clearance was decreased by cortisol and epinephrine infusions in both diabetics and controls. The diabetics in this study were given continuous insulin infusion during these hormone challenges, so that the plasma glucose elevations occurred in spite of insulin replacement.

Twenty-four hour integrated plasma concentrations of aldosterone, norepinephrine, epinephrine and growth hormone were all found to be significantly higher in diabetics than in nondiabetics (Zadik, Kayne, Kappy, Plotnick, & Kowarski, 1980). Diabetics in this experiment had received their daily insulin. Plasma norepinephrine and epinephrine

measurements at a single time point have not demonstrated differences between diabetics and nondiabetics (Christensen, 1974; Gustafson & Kalkhoff, 1981) as did integrated 24 hour measures (Zadik et al., 1980). However, diabetics experience excessive increments in plasma glucose as well as in plasma epinephrine upon standing from a sitting position, or during recovery from isometric exercises (Gustafson & Kalkhoff, 1981).

Animals with experimentally induced diabetes also have been shown to experience alterations in peripheral concentrations of both corticosteroids and catecholamines. Streptozotocin diabetic rats had elevated corticosterone levels during the afternoon from about 1 P.M. to 6 P.M. (with lights out at 8 P.M.), with a general flattening of the normal day-night rhythm (Tornello, Coirini, & De Nicola, 1981b). In addition, residual pituitary ACTH activity under resting conditions, as measured in a bioassay, was reduced in diabetics relative to controls (De Nicola, Fridman, Del Castillo, & Foglia, 1976, 1977). In response to the stress of IP injection of cold water, residual ACTH activity was reduced in the control rats but remained at the same level in diabetic rats as during basal conditions (De Nicola et al., 1977). Following withdrawal of insulin replacement in alloxan diabetic rats, morning corticosterone concentrations were elevated and the adrenocortical response to ACTH was increased (L'Age, Langholz, Fechner, & Salzmann, 1974).

There has been considerably less investigation of catecholamines in animal models of diabetes. Thus, little has been published concerning sympathetic and adrenomedullary function in the diabetic rat. However, a recent report noted decreased norepinephrine turnover in interscapular brown adipose tissue, heart and pancreas of streptozotocin diabetic rats 9 weeks after diabetes induction (Yoshida, Nishioka, Nakamura, & Kondo, 1985).

Another change noted in the streptozotocin-diabetic rat was an increase in plasma dopamine-beta-hydroxylase (DBH) activity (Berkowitz & Head, 1978; Berkowitz, Head, Joh, & Hempstead, 1980; Schmidt, Geller, & Johnson, 1981). This enzyme converts dopamine to norepinephrine, and thus is related to synthesis of both norepinephrine and epinephrine. The DBH elevations occurred within 24 hours of streptozotocin administration (Schmidt et al., 1981) and remained elevated when measured 7 months after diabetes induction (Berkowitz et al., 1980). Plasma norepinephrine was also found to be elevated in diabetic rats 4 weeks, but not 16 weeks, after streptozotocin treatment. At 9 weeks, diabetic rats had reduced plasma concentrations of both norepinephrine and epinephrine despite the continuing elevation in DBH (Berkowitz & Head, 1978). Clearly, changes in peripheral catecholamine systems occur in the diabetic rat.

A number of central nervous system (CNS) changes have also been found in diabetic rats. For example, streptozotocin diabetic rats had elevated NE concentrations in hypothalamus and midbrain, decreased concentrations of DOPAC and 5-hydroxyindole-acetic acid (5HIAA) in thalamus and midbrain, increased alpha-adrenergic receptor concentration (maximum binding capacity) in medial hypothalamus and midbrain, and decreased $V_{\max}$ of tyrosine hydroxylase in hypothalamus, thalamus, medulla and midbrain (Bitar, Koulu, Rapoport, & Linnoila, 1986). Brain serotonin synthesis is also reduced in diabetic rats (Crandall & Fernstrom, 1983). There are alterations in brain adenylate cyclase activity, including reduced sensitivity to stimulation by catecholamines, as well (Palmer, Wilson, & Chronister, 1983).

Increased forebrain NE and decreased 3-methoxy, 4-hydroxyphenylethylene glycol (MHPG) concentrations in diabetic rats were normalized with insulin treatment (Trulson & Himmel, 1985). Dopamine synthesis in striatum and limbic forebrain is also decreased in diabetic rats, and dopamine receptor number in striatum increased (Lozovsky et al., 1981; Trulson & Himmel, 1983; Saller, 1984; Serri, Renier & Somma, 1985).

There was a decrease in the maximum number of binding sites ($B_{\max}$) for [^3H]-corticosterone in hypothalamus and hippocampus of diabetic rats, while no changes in

corticosterone binding were noted in pituitary or cerebral cortex (Tornello, Fridman, Weisenberg, Coirini, & De Nicola, 1981a).

Clearly both humans and animals with diabetes experience a number of changes in peripheral concentrations of both steroids and catecholamines. In diabetic rats there are also a large number of alterations in corticosteroid and monoamine function in the central nervous system as well. However, whereas there is a substantial clinical literature concerning behavior/hormone interactions in diabetes, there is almost no information about behavioral changes in diabetic animals.

One behavioral deficit has been reported in the diabetic rat. Stereotypy induced by dopaminergic agonists was attenuated in the diabetic rat (Marshall, 1978; Bellush & Rowland, in press). Both the duration and intensity of stereotyped sniffing and oral behaviors induced by peripheral injection of both apomorphine and amphetamine were affected. It is quite possible that the attenuation seen in the diabetic animals following amphetamine administration is related to the reported decrease in dopamine synthesis in limbic forebrain (Trulson & Himmel, 1983) and striatum (Lozovsky et al., 1981). However, apomorphine-induced stereotypy would be expected to be increased in diabetics, given the increased number of dopamine receptors in these rats. Altered uptake of

apomorphine into brain (Saller, 1984) or alterations in adenylate cyclase responsivity to catecholamine stimulation (Palmer et al., 1983) may account for this latter finding. In any case, we were unable to normalize stereotyped behavior in diabetic rats with dietary enrichment with tyrosine, the amino acid precursor of dopamine, which nonetheless increased brain tyrosine concentrations (Bellush & Rowland, in press). While this attenuation of apomorphine- and amphetamine-induced stereotypy in diabetic rats is a heuristic tool for investigation of diabetes-related behavioral changes, it has a limited applicability to other behaviors because it is a pharmacologically induced behavior. However, it does suggest that the neurochemical alterations in diabetes may lead to functional consequences.

Summary

Given the numerous hormonal and neurochemical changes which have been documented in diabetics, as well as the vast clinical literature suggesting potential interactions of hormones and behavior, it is somewhat surprising that no investigations of cognitive performance in animal models of diabetes have yet been reported. To our knowledge, the only learning paradigm which included diabetic rats studied the effects of insulin-induced hypoglycemia upon appetitive learning and included an insulin treated diabetic group only for the purpose of comparison, and not as a focus of the study (Clayson, 1971). However, her results indicated that

insulin-treated diabetics learned an appetitive T-maze task more quickly than untreated nondiabetic rats, while insulin-treated diabetics failed to learn the task at all.

Motivational variables cannot be ruled out in this study, since water deprivation was involved, and diabetic rats are known to be hyperdipsic.

The following experiments were conducted to evaluate possible changes in learning and memory in diabetic rats. Because stress has been reported to be a major factor in behavior/hormone interactions in the clinical literature, the present studies utilized a stressful (aversive) learning paradigm. This involved learning to avoid a place where footshock punishment was delivered. Acute stresses associated with a learning situation have been evaluated in human diabetics (Kemmer et al., 1986).

In addition to the evaluation of a short-term stress, experiments were conducted to compare the hormonal and neurochemical effects of 72 hours of continuous exposure to a cold environment (4°C) in diabetic and nondiabetic rats. The parallels to this chronic stress situation in the clinical literature are adolescence and the period of proliferation of retinopathy. Both of these periods represent developmental periods when stress interacts for extended lengths of time with metabolic and behavioral variables in diabetes management. As with all behavior/stress interactions in diabetes mellitus, it is

difficult to determine whether compliance problems are the primary cause of hormonal changes and metabolic instability, or whether some physiological factor first precipitates behavioral problems. While no behavioral comparisons were made in connection with chronic stress in the present investigations, a number of physiological responses to cold stress were compared in diabetic and nondiabetic rats. In addition, some diabetic and nondiabetic rats were given daily insulin injections so that it could be determined if insulin treatment in diabetic rats would normalize any altered responses found in untreated diabetics.

CHAPTER II
COMPARISON OF ACTIVITY AND REACTIVITY
IN DIABETIC AND NONDIABETIC RATS

Introduction

Chapter I outlined a large and diverse literature concerning hormone/behavior interactions which occur both in human diabetes and in experimentally induced diabetes in animals. It was also emphasized that the hormones disturbed by diabetes are the so-called stress hormones, and that these hormones are importantly involved in the mechanisms of memory formation and retrieval. Given these alterations in diabetes mellitus, it is somewhat surprising that little attention has been given to the investigation of learning and memory in animal models of diabetes. The experiments in this chapter were designed to investigate possible alterations in either learning or memory in streptozotocin-diabetic rats. They involved a comparison of retention for one-trial passive avoidance training in streptozotocin-diabetic and nondiabetic rats. An aversive task was chosen because the stress hormones are more clearly involved in such a task than in appetitive learning situations and because motivation for food- or water-reinforced learning

might differ in diabetics, which are both hyperdipsic and hyperphagic.

Before investigating learning and memory in the diabetic rat, it was necessary to determine whether there would be either motivational or perceptual differences between diabetic and nondiabetic rats which would be mistaken for associative differences. There is some basis for expecting perceptual differences as a result of neuropathy which could be present in diabetic rats (Jakobsen, 1978; Clements, 1979). Such neuropathy could result in reduced sensitivity to footshock, the unconditioned stimulus to be used in training, in the diabetic rats. Since retention of passive avoidance is related to intensity of footshock (Ader, Weijnen, & Moleman, 1972), diabetic rats might appear to be impaired in their ability to learn the task when, in fact, they simply perceive the stimulus as less intense. Experiment 1 addressed this possibility by comparing flinch-jump thresholds of diabetic rats to those of nondiabetic rats.

Another potential problem in comparing diabetics to nondiabetics involves possible differences in general activity levels. Thus, utilization of metabolic substrates for energy production is compromised in diabetic rats, and they may therefore be less active than nondiabetics. Since the learning task to be employed was a one-trial passive avoidance task, inactivity could be mistaken for better

learning. This possibility was to be evaluated in the learning paradigm itself by measurement of each animal's latency to enter the learning chamber both prior to footshock training and after footshock training. Still, the issue of differential activity levels in diabetic and nondiabetic rats can be more carefully determined by a direct test. Experiment 2 evaluated diabetic and nondiabetic rats in open field activity tests, which are designed to reflect both activity and emotionality in a novel environment (Veldhuis & deWied, 1984).

Experiment 1. Flinch-Jump Thresholds
of Diabetic and Nondiabetic Rats

A longstanding procedure for determining the sensitivity (or responsivity) of rats to footshock is that of the flinch-jump threshold test (Evans, 1961). It employs the method of limits used in a number of psychophysical measurements of sensory systems. Briefly, stimuli of varying intensity are presented in alternating ascending and descending series and the behavioral response is recorded. If diabetic rats have a reduced capacity to respond to footshock because of neuropathy, their flinch-jump thresholds would be expected to be higher than those of nondiabetics and it would be necessary to adjust the footshock intensity used in passive avoidance training in order to equate the responsivity of diabetics with that of nondiabetics.

Another factor of concern in the learning situation is the effect of glycemic state itself (Gold, in press). In testing for such effects in the passive avoidance task, some rats were to be given insulin during either training or test sessions. Insulinization might influence the sensitivity of diabetics or controls, or both, to footshock. Thus, in the flinch-jump experiment, groups of insulin-treated diabetic and control rats were included.

A final consideration in attempting to equate the responsivity of diabetic and nondiabetic rats was the disparity in body weight, often 100-200g, between diabetic rats and age- and treatment-matched controls. Marks and Hobbs (1972) found heavier rats to be significantly less responsive to footshock than lighter animals (but see also Beatty & Beatty, 1970). To rule out such a problem, a group of nondiabetic rats which weighed the same as diabetic rats was tested.

Methods

Animals and housing

All rats were adult male Sprague Dawley albino rats (Zivic Miller, Pittsburgh). They weighed 350-425 g at the beginning of the experiment. They were housed individually in hanging wire cages in a vivarium maintained at $21 \pm 2^{\circ}\text{C}$ and an artificial 12:12 hour light/dark cycle (lights on at 7:00 A.M.). Purina Laboratory Chow (Purina #5001) and water were available ad libitum at all times unless otherwise stated.

Induction of diabetes

Diabetes was established in 20 rats by intraperitoneal (IP) administration of 65 mg/kg streptozotocin (STP) in citrate buffer, 0.1M, pH=4.5. Twenty additional rats were given the citrate buffer. The remaining 9 rats were untreated and were tested when they reached the same body weight as diabetic rats (which typically weigh 100-200 g less than their age- and treatment-matched controls). Diabetes was confirmed 1 week after STP treatment by testing the urine for glucose with Ketodiastix (Ames division of Miles Laboratories). STP-treated rats which did not show any glucose in the urine were reinjected at this time. Only a few rats needed to be reinjected, and all the rats treated with STP became severely diabetic within 2-3 weeks (blood glucose concentrations > 400 mg/dl). One month after STP administration, insulin replacement was begun in 10 diabetic and 10 control rats. This consisted of 3 days of twice daily (8:00 A.M. and 5:00 P.M.) subcutaneous (sc) injections of 1 UNIT (U) protamine zinc insulin (PZI) followed by 7 days of twice daily injections of 2.5 U PZI.

Apparatus

Testing was conducted in an 8 in x 9 in x 8 in plexiglas chamber (This was actually one side of a 2 compartment shuttle box, the other half of which was not used in this experiment). It had a grid floor of steel rods spaced 7/16 in apart. The grids were wired to a constant current

shocker scrambler (Lehigh Valley Electronics) and a 28 V power supply (Lambda). A universal timer (Lehigh Valley Electronics) controlled the duration of the shocks. A 25 W light bulb was suspended 18 in above the chamber, providing the only light in the room during testing.

Procedure

All testing was conducted between 1:00 P.M. and 5:00 P.M. A rat was placed into the chamber and allowed 30 sec to explore and to adapt to the new environment. At that time, 10 series of 0.5 sec shocks were administered in alternating ascending and descending sequence. The current levels (in mA) ranged from 0.02 to 0.1 mA in 0.02 mA steps, and from 0.1 mA to 1.0mA in 0.1 mA steps for a total of 13 shocks in each series. Two observers independently rated the response of the rat to each shock as follows (Mactutus & Tilson, 1984): 0--No response; 1--Flinch: contraction of any part of the body; 2--Flinch-shuffle: flinch plus movement of one paw off the grid; 3--Shuffle: movement of two or three paws off the grid; 4--Shuffle-jump: movement of paws plus animal startles; 5--Jump: all paws leave grid simultaneously or animal moves position at offset of shock. All the scores of both observers were tabulated to produce a frequency of occurrence of each behavioral response category for each rat. Responses 2 (flinch-shuffle) and 4 (shuffle-jump) were distributed equally between the categories to either side, (e.g. responses 2 were equally distributed to response

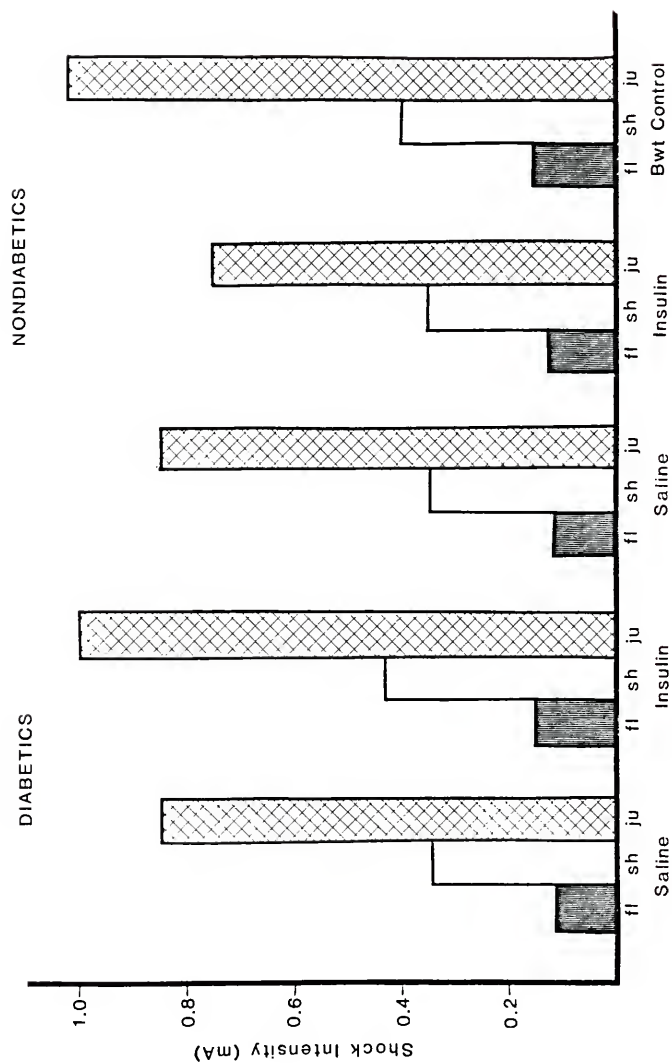
categories 1 and 3). Flinch, shuffle and jump thresholds were defined as the lowest current level (interpolated) to which the response occurred on 50% of the trials. Animals not reaching the 50% criterion for jump threshold at 1.0 mA were assigned a threshold of 1.1 mA. The 3 thresholds were determined for each subject. Median threshold scores were then tabulated for each group--controls, insulin-treated controls, diabetics, insulin-treated diabetics, and weight matched controls. Flinch, shuffle and jump thresholds for the 5 groups were compared with Kruskal Wallis analysis of variance by ranks.

At the end of the flinch-jump testing session, blood was taken from each rat by making a small nick in the tail with a scalpel blade and stroking the tail to collect blood in heparinized micro-hematocrit capillary tubes (Fisher Scientific). Plasma glucose concentration was determined, after centrifugation, with a Yellow Springs Instruments 23A automated glucose analyzer. Mean plasma glucose concentrations and mean body weights of the 4 age- and treatment-matched groups were compared with analysis of variance (ANOVA). Mean body weight of the body weight control group was compared with that of saline treated diabetics with a t-test.

Results and Discussion

Figure 1 shows the median threshold scores for the 5 groups. There were no significant differences among any of

Figure 1. Median flinch (fl) shuffle (sh) and jump (ju) thresholds of saline-treated and insulin-treated diabetic and nondiabetic rats and of nondiabetic rats matched to saline-treated diabetics in body weight (Bwt Control).



the groups on any of the thresholds (Kruskall-Wallis H values were flinch-1.7; shuffle-3.92; jump-7.62; $P_s > 0.10$).

Figures 2 and 3 show mean body weights and mean blood glucose concentrations respectively of the 5 groups. Body weights varied significantly with main effects of diabetes ($F(1,36)=118.12$, $P<0.001$) and of insulin replacement ($F(1,36)=15.91$, $P<0.001$), as well as an interaction ($F(1,36)=4.84$, $P<0.05$). Thus, the diabetic rats weighed significantly less than did the nondiabetic rats, and insulin replacement led to significantly higher body weight in diabetic rats but did not affect the weight of nondiabetics. As intended, the weight of the younger body weight control group did not differ significantly from that of the saline-treated diabetics ($t(17)=1.77$, $P>0.05$). Plasma glucose concentrations also varied significantly with main effects of diabetes $F(1,36)=172.55$, $P<0.001$) and of insulin replacement ($F(1,36)=203.75$, $P<0.001$), as well as an interaction ($F(1,36)=142.22$, $P<0.001$). That is, insulin-treated diabetic and nondiabetic rats had significantly lower blood glucose concentrations than did their saline-treated counterparts. Insulin-treated diabetic rats had blood glucose concentrations indistinguishable from those of nondiabetic rats, while the saline-treated diabetic rats had significantly higher blood glucose concentrations.

No differences occurred in any of the thresholds measured in experiment 1 on the basis of diabetes, insulin

Figure 2. Mean body weights of of nondiabetic and diabetic groups at the time of footshock threshold determinations. (sal=saline-treated, ins=insulin-treated, bwt=body weight controls).

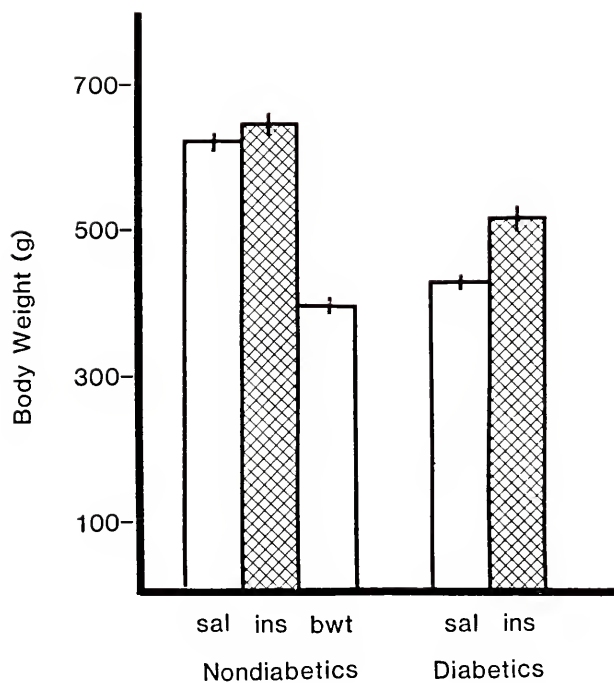
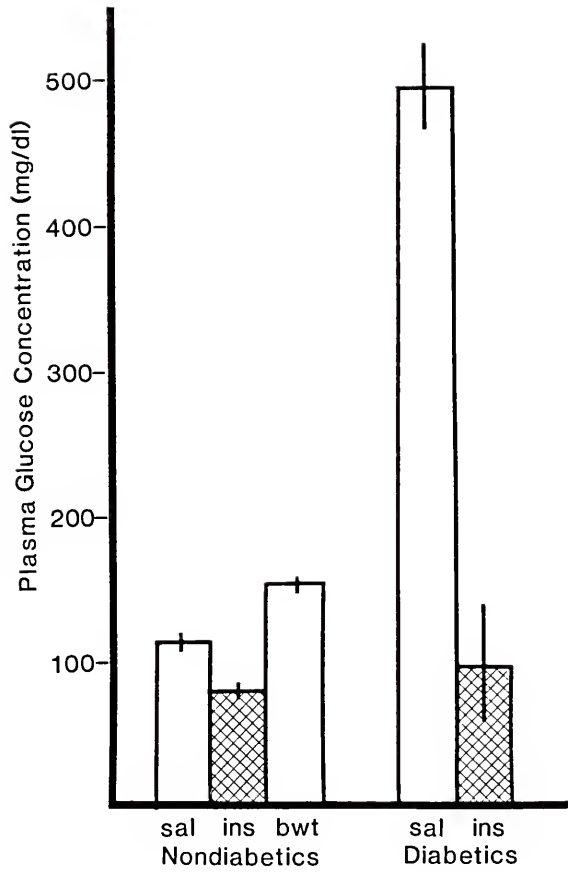


Figure 3. Mean plasma glucose concentrations of nondiabetic and diabetic groups measured immediately following footshock threshold determinations (sal=saline-treated, ins=insulin-treated, bst=body weight controls).



administration or body weight. In contrast to the findings of Marks and Hobbs (1972), the present study did not detect a difference in sensitivity to footshock in lighter versus heavier control rats. Diabetics would therefore not be expected to differ from controls in sensitivity to footshock on the basis of differences in body weight. Further, the fact that the diabetics did not differ from controls suggests that any neuropathy existing in the diabetics had no measurable effect upon responsivity to footshock.

Finally, in spite of a wide range of plasma glucose concentrations from hypoglycemic to hyperglycemic ranges, no difference in thresholds to footshock occurred. These results made it possible to proceed with the learning experiments with the assurance that any differences in passive avoidance responding which might be found in diabetic versus nondiabetic rats would not be the result of differential responsivity to footshock, but could confidently be ascribed to the effects of training.

Experiment 2. Open-Field Activity of Diabetic and Nondiabetic Rats

Differences in either emotionality or activity level in diabetic and nondiabetic rats when placed in a novel environment might also lead to differences in performance of passive avoidance unrelated to associative processes. The passive avoidance paradigm to be used later was planned to include measures of latency to enter the shock chamber prior to footshock training; but it was necessary to evaluate

diabetic animals and determine prior to employing the passive avoidance task whether differences in levels of activity in diabetic and nondiabetic rats might produce spurious differences in passive avoidance performance. To evaluate potential differences in general activity levels of diabetic and nondiabetic rats, an open field arena was employed (Britton, Koob, Rivier, & Vale, 1982).

Methods

Subjects. Subjects were 50 adult male Sprague Dawley rats, 27 of which had been made diabetic with streptozotocin (STP), exactly as in Experiment 1. The remaining 23 rats had been injected with vehicle. Beginning thirty days after STP or vehicle injections, 14 diabetic and 6 control rats were treated with insulin for 10 days, while the remaining rats received injections of the vehicle as described in Experiment 1.

Apparatus. The open field was a square enclosure, consisting of 4 plywood walls measuring 27 in long by 14 in high held together with L-braces and open at both top and bottom. This open cube was placed on a white table upon which were painted a black outline measuring 27 in on each side and 9, 9-in black squares. Indirect lighting was provided by an overhead light in another part of the room.

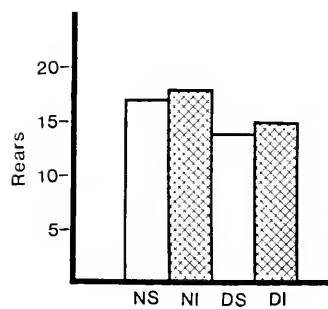
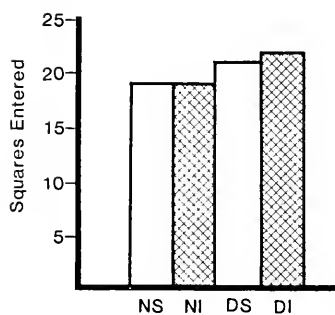
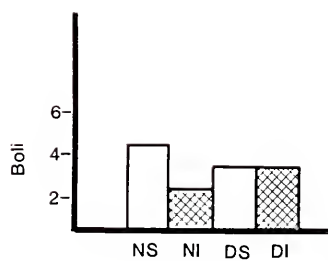
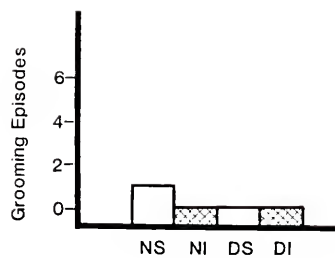
Procedure. The animals tested in the open field were those to be trained in passive avoidance. Two days prior to passive avoidance pretraining, each animal was brought from

the vivarium in its home cage and placed in the center square of the open field. A running timer was activated and the animals were observed for 3 minutes by two observers. One observer counted and recorded the number of squares the animal entered. The other observer recorded the number of rears (i.e. raising up on hindlimbs), the number of grooming episodes and the number of fecal boli. After each subject was tested, the field was cleaned with 70% alcohol. Because there were disparate numbers of subjects in the various groups (17 insulin-treated controls, 6 saline-treated controls, 7 insulin-treated diabetics and 7 saline-treated diabetics), median scores were determined for each behavioral category for each of the 4 groups. Kruskal-Wallis analysis by ranks was used to evaluate the median scores.

Results and Discussion

Results of the open field activity measurements are shown in Figure 4. There were no significant differences among the groups in any of the behavioral categories ($P > 0.10$). In these measures of activity and emotionality upon exposure to a novel environment, there were no differences between diabetics and nondiabetic controls. This finding in the open-field test was in agreement with a previous finding in this laboratory (Rowland, Joyce, & Bellush, 1985) using a photocell activity-monitoring cage. Under these conditions, diabetics were indistinguishable from controls during the

Figure 4. Median number of grooming episodes, boli excreted, squares entered and rears in saline-treated and insulin-treated nondiabetic and diabetic rats (NS=nondiabetic saline-treated, NI=nondiabetic insulin-treated, DS=diabetic saline-treated, DI=diabetic insulin-treated).



initial 5 minute exposure to the environment, but became less active than controls during the latter portion of the 30 minute session. Both the photocell activity monitoring results and the present results in the open field argue against any activity or emotionality differences between diabetic and nondiabetic rats in short term measurements. Thus, there should not be a problem with inactivity in the passive avoidance procedure, which entails evaluation of behavior for a total of 10 minutes. Indeed, animals failing to move from a lighted chamber into a darkened chamber (where shock would later be delivered) in the first minute of exposure to the shuttle box before footshock training was given would be excluded from further testing. The results of the present study and of the previous study in this laboratory indicate that systematic activity differences in diabetic rats would be very unlikely to interfere with the evaluation of passive avoidance learning.

CHAPTER III
ONE-TRIAL PASSIVE AVOIDANCE LEARNING
IN DIABETIC AND NONDIABETIC RATS

Introduction

The clinical literature cited in Chapter I indicated that a number of cognitive impairments may accompany diabetes mellitus. In addition, a number of reports were cited which documented changes in metabolic response to catecholamine or cortisol challenge (Shamoon et al., 1980) and elevated cortisol concentration in plasma of some diabetics at certain times (Lebinger et al., 1983), although the relationship of these hormonal alterations to cognitive and psychological changes found in some diabetic individuals remains to be clarified. It has been difficult to determine whether cognitive and emotional alterations reported in diabetics are in any way the result of hormonal and metabolic changes, or whether the behavioral phenomena are psychologically based. Negative attitudes toward the restrictive lifestyle imposed by diabetes could evoke both behavioral problems and problems of compliance which would secondarily cause hormonal derangement. Such complexities can be avoided by turning to animal models of diabetes for further clarification of the relationship between hormonal

changes occurring in diabetes and changes in cognitive function.

In experimentally induced diabetes in rats, there are alterations in concentrations of corticosterone in plasma, both in basal and in stimulated states, and elevations in plasma concentrations of DBH and norepinephrine, as well as numerous functional changes in central nervous system (CNS) monoamine systems. Moreover, the importance of both peripheral and central actions of hormones of the hypothalamo-pituitary-adrenal system and of the adrenomedullary system in learning and memory in animals have been demonstrated convincingly (Dunn & Kramarcy, 1984).

What remain to be investigated are the possible changes in learning and memory in animal models of diabetes, given that the hormonal systems affected by diabetes are those clearly involved in processing of memory. The following experiments deal with the evaluation of learning and memory, as well as some related hormonal and neurochemical measurements, in the streptozotocin diabetic rat.

The experimental learning paradigm selected for these studies is one-trial passive avoidance, in which learning is restricted to a specific point in time. It thus provides an opportunity to investigate some of the physiological events which accompany either the training episode or the retention test. Specifically, these experiments compared retention for a one-trial passive avoidance task in diabetic and

nondiabetic rats. In addition, plasma corticosterone concentrations, which are thought to reflect stressful stimuli (Bassett & Cairncross, 1975), as well as influence learning and memory (Cottrell & Nakajima, 1977), were measured following training and testing sessions, as were regional CNS monoamine concentrations following the retention test. Twenty-four hour urinary catecholamine excretion, a measure of peripheral catecholamine activity (Roy, Sellers, Flattery, & Sellers, 1977), was measured during a baseline period and following footshock.

Experiment 3. Retention for Passive Avoidance Training in Diabetic and Nondiabetic Rats

This experiment was divided into two separate manipulations. In Experiment 3a, state-dependent retention, based upon chronic changes in glycemic state, was evaluated. Chronic (10 day) administration of insulin was utilized because it corresponds with the duration of replacement reported to normalize diabetes-induced increases in striatal dopamine receptor number (Lozovsky et al., 1981; Serri et al., 1985; Trulson & Himmel, 1983).

If state-dependent effects were related in any way to known alterations in neurochemistry in the diabetic, such as monoamine synthesis or change in receptor number, an insulin replacement regimen which has been shown to normalize such changes might also optimize any state dependent phenomena. State-dependent effects were evaluated in both diabetic and nondiabetic rats. The glycemic changes produced by insulin

are much more dramatic in diabetics, and insulin replacement identical to that in diabetic rats did not alter dopamine receptor number in striata of nondiabetic rats (Lozovsky et al., 1981). However, insulin administration affects many systems, any of which may be involved in state-dependency. Thus, insulin may support state dependent retention in both diabetic and nondiabetic rats. Alternatively, if diabetics were subject to state-dependent effects while nondiabetics were not, then the central dopaminergic alterations and large excursions of plasma glucose concentration of diabetes would be implicated in these behavioral effects. The inclusion of nondiabetic rats along with diabetic rats in this experiment thus would help to clarify the mechanisms underlying state-dependency.

As noted in the introduction there was a report of impaired cognitive performance in diabetics at high and low plasma glucose concentrations (Holmes et al., 1983), while another report demonstrated superior performance by diabetics when either hyperglycemic or normoglycemic (Flender & Lifshitz, 1976). In a study with rats, hypoglycemia prevented the achievement of performance to criterion in an appetitive T-maze task (Clayson, 1971). In this study, insulin-treated diabetic rats learned the task more quickly than normoglycemic control rats. Thus, results of previous studies are conflicting as to whether diabetes impairs or enhances performance. Indeed, different tasks

may lead to different results. The present experiment was designed to examine retention for a relatively simple, one-trial aversive task. One objective was to determine whether retention for the task would be different if the glycemic state during testing was the same as, or different from, the glycemic state during training.

In addition to producing state-dependent effects upon retention, diabetes could influence memory storage and retrieval mechanisms as a result of producing alterations in peripheral and central catecholamine and steroid activity outlined in Chapter 1. In Experiment 3b, this possibility was investigated. A method commonly used to evaluate hormone-behavior interactions is that of assessing both behavioral performance and the associated hormonal and neurochemical changes, at different levels of footshock (e.g., McCarty & Gold, 1981). In the present experiment, separate groups of diabetic and nondiabetic rats were given 1- and 2-second shocks and compared for retention, as well as for a number of physiological variables reported to be sensitive to aversive learning. Longer durations rather than higher intensity shocks were employed, because the passive avoidance task involved 1.0 mA shocks throughout the state-dependent experiment. This shock intensity was the maximal intensity evaluated in flinch-jump testing in Experiment 1. There was no way to assure that shocks of higher current intensity would affect diabetics and

nondiabetics equally. However, it has been shown that retention improves as a function of duration of shock as well as its intensity (Ader et al., 1972).

Experiment 3a. State-Dependent Effects of Glycemic State

Methods

Subjects. Subjects were 116 adult male Sprague Dawley rats weighing 250-400 g at the beginning of the experiments. They were housed singly in hanging wire cages in a vivarium maintained at $21 \pm 2^{\circ}\text{C}$ with a 12:12 hour light:dark cycle (lights on at 7:00 A.M.). Purina lab chow and water were freely available at all times.

Induction of diabetes. Diabetes was induced in half the rats with administration of streptozotocin exactly as described in Experiment 1, while the remaining rats were given vehicle.

Insulin replacement. Thirty days after induction of diabetes, rats were assigned to groups for the passive avoidance task. There were 4 groups of control rats: one group was to receive saline throughout all training and testing (SS); the second group (SI) was to receive saline for 10 days prior to passive avoidance training. On the evening of the training session, these rats began receiving insulin for 10 days. The first 3 days they received 1 UNIT (U) of protamine zinc insulin (PZI) twice daily and the next 7 days they received 2.5 U PZI twice daily. The third group (IS) received insulin as just described for 10 days prior to

training and were reversed to saline the evening of training. The fourth group of controls was a departure from the usual symmetrical design in state dependent learning in which they would have received insulin throughout training and testing. It was decided instead to train this group of rats (SSTP) while normoglycemic and inject them during the evening of training with STP so that they were hyperglycemic at testing 10 days later.

Diabetic groups included SS, SI and IS identical to the control groups with the fourth diabetic group (II) receiving insulin throughout. Thus, in the diabetics a symmetrical state-dependent design was employed.

Criteria for defining glycemic states were established as follows. In diabetics the insulin treatment was required to provide plasma glucose concentrations of less than 200 mg/dl during the training and/or testing procedure. Any insulin-treated diabetic rats with concentrations of glucose higher than this value were eliminated from the study. In the controls, it was required only that plasma glucose concentrations of the animals in the SI and IS groups be lower during the insulin treatment than when saline was given.

Apparatus. Passive avoidance training was conducted in a clear plexiglas two-chamber shuttle box. Both chambers measured 8 in x 9 in x 8 in high. The clear side walls of one chamber were covered with white paper, while those of

the other were covered with black paper. The grid floor of the white chamber was covered with a clear plexiglas sheet. A clear plexiglas lid was placed over the white chamber when the rat was inside the shuttle box, and a 25 W bulb was suspended 18 in above this chamber. A mirror was placed at an angle above this chamber so that the activity of the rats could be observed while the experimenter remained unnoticed. A plywood lid was placed on top of the dark chamber. The grid floor of the dark chamber was wired to a constant current shocker-scrambler as described in Experiment 1. The two chambers were separated by a guillotine door which was operated manually by the experimenter. The differences between the two shuttle box chambers served two purposes. First, they maximized the distinctiveness of the dark chamber, where the rat was to be shocked, from the white chamber, which was "safe." Second, the white, or "safe," chamber was designed to be brighter, and thus initially less preferred by, or even slightly aversive to, the rats. Footshock training was therefore designed to overcome a natural aversion to the lighted chamber.

Procedures. Due to the large number of animals involved in Experiment 3, the study was conducted in four separate replications, in each of which animals from all the groups were represented. In addition, in each of the replications, the rats were divided into squads, each containing equal numbers of diabetic and nondiabetic rats. All training and

testing sessions were conducted in the afternoon, between 1:00 and 5:00 P.M. After the initial ten days of administration of either insulin or saline, each rat was given a pretraining trial which consisted of a 10-minute exposure to the shuttle box. Briefly, the rat was placed into the white chamber, facing away from the guillotine door, and a 10-minute timer was activated. Ten seconds later, the guillotine door was opened and a hand-held stopwatch was activated. The latency of the rat to enter the dark chamber was recorded on the stopwatch. During the remainder of the 10-minute session, the stopwatch was activated whenever the rat was in the white chamber, and the total time spent in this chamber (TTW) was also recorded.

Twenty-four hours after pretraining, the rats were again placed into the white chamber of the shuttle box, the guillotine door was opened and the stopwatch activated. When the rat entered the dark chamber, latency to enter was again noted. The door was then closed, confining the rat to the dark chamber, and four 1-second, 1 mA footshocks were delivered at approximately 10-second intervals, beginning 5 seconds after the door was closed. Within 5 seconds after the last shock, the rat was removed from the dark chamber of the apparatus and returned to the vivarium. Exactly 15 minutes later, a blood sample was collected from a tail nick into heparinized micro-hematocrit capillary tubes, centrifuged for three minutes and the plasma separated and

frozen until determinations of plasma glucose and corticosterone concentrations were made. On the evening after this training, those animals whose injections were to be reversed were started on the new regime. Those control rats who were to receive STP were given these IP injections and then continued on twice daily saline.

Ten days after the training trial, each rat was again placed into the white chamber of the shuttle box, and a 10 minute session identical to the pretraining trial was conducted, with latency to enter the dark chamber and total time in the white chamber once again recorded. At the end of the 10 minute retention trial the rat was placed in a holding cage in the training room for an additional 5 minutes to allow a total of 15 minutes to elapse after exposure to the fear cues induced by the reexposure to the shuttle box.

At this time the rat was taken to another room and sacrificed by decapitation (the rats could not be anesthetized in any way prior to decapitation, because this would have interfered with many of the biochemical measurements to be made). Trunk blood was collected in heparinized 15 ml tubes and kept on ice until it was centrifuged for 10 minutes. Plasma was collected and frozen for later determination of plasma glucose and corticosterone concentrations. The brain was quickly removed and placed on ice, and the following regions were dissected and frozen at

-70°C for later determinations of monoamine and metabolite concentrations: frontal cortex, hypothalamus, amygdala, hippocampus and brainstem. Plasma corticosterone concentrations and neurochemical analyses will be discussed in Experiments 4 and 6 below.

Data analysis. Body weights and plasma glucose concentrations were compared with one-way analysis of variance (ANOVA) and Duncan's post hoc evaluations of individual differences. Pretraining and training latencies for each rat were averaged and these average latencies were considered training scores. Latencies and TTWs were tested with Kruskal-Wallis one-way nonparametric analysis by ranks.

Results and discussion

Tables 1 and 2 show plasma glucose concentrations and body weights of all the groups both at the time of training and at the time of retention testing. As expected, diabetics not receiving insulin had plasma glucose concentrations well over 400 mg/dl, while insulin-treated groups had concentrations in the normal range. Plasma glucose concentrations of insulin-treated nondiabetic rats were about half that of non-insulinized groups, and STP treatment produced increments to severe hyperglycemic values (SS2 groups will be described in Experiment 3b, below).

Body weights also followed expected patterns, with diabetics weighing substantially less than nondiabetics. Insulin replacement led to normalized weight gain in

TABLE 1

Plasma Glucose Concentrations of Rats at
the Time of Passive Avoidance Training
and Retention Testing

<u>Group</u>		<u>Plasma Glucose (mg/dl)</u>	
		<u>Training</u>	<u>Testing</u>
<u>Nondiabetics</u>	SS	131+4	146+3
	SI	142+5	76+7 ⁺
	IS	62+7 ⁺	142+6
	SSTP	135+2	449+27 ⁺
	SS2	141+5	151+3
<u>Diabetics</u>	SS	568+29*	554+29*
	SI	630+53*	93+12
	IS	119+13	470+21 ⁺
	II	93+11	90+9
	SS2	541+17*	549+29*

Shown are $\bar{X} \pm \text{SEM}$

* Significantly different from IS and II

⁺ Significantly different from all other groups

TABLE 2

Body Weights of Rats at the Time of
Passive Avoidance Training and Retention Testing

<u>Group</u>	<u>Bodyweight (g)</u>	
	Training	Testing
<u>Nondiabetics</u>	SS	497+9
	SI	532+8
	IS	534+12
	SSTP	545+12
	SS2	515+12
		473+13 ⁺
		561+10
<u>Diabetics</u>	SS	375+17*
	SI	375+17*
	IS	407+17
	II	442+15
	SS2	412+10
		476+10
		376+7*
		383+11*

Shown are $\bar{X} \pm \text{SEM}$

* Significantly different from IS and II

⁺ Significantly different from all other groups

diabetics but produced no apparent effects in nondiabetics. The SSTP group lost weight between training and testing. This was an expected effect of the STP treatment.

The behavioral results are shown in Table 3. Kruskal-Wallis analysis by ranks comparing training and test latencies and total time spent in the white chamber (TTW) revealed no significant differences among groups in either the diabetic or nondiabetic state-dependent retention manipulations ($P_s > 0.1$). Thus, despite large changes in blood glucose concentrations from training to testing in several of the groups, there was no decrement in retention of the footshock training in these groups relative to groups whose glycemic state was not changed.

It was somewhat surprising that dramatic changes in glycemic state, which were likely accompanied by numerous other metabolic changes as well, failed to provide the basis for state-dependent retention of the passive avoidance task. In any case, on the basis of these negative results it might therefore be concluded that the notion of state-dependency is of little explanatory value.

However, two factors may have interfered with the establishment of state dependency in the present study. First, it was established in a pilot experiment that in order to obtain robust retention in untreated rats over a 10 day period, it was necessary to deliver four footshocks in the training session. It is, however, possible that this

TABLE 3

Latencies to Enter the Dark Chamber and Total Time
Spent in the White Chamber (TTW) of State Dependent
Nondiabetic and Diabetic Groups

<u>Group</u>	<u>Pre-Training</u>		<u>Post-Training</u>	
	Latency	TTW	Latency	TTW
<u>Nondiabetics</u>	(sec)	(sec)	(sec)	(sec)
SS	7.0	34.7	114.2	302.9
SI	8.4	101.2	438.0	569.9
IS	10.3	46.8	166.3	367.5
SSTP	8.6	109.9	481.4	580.9
<u>Diabetics</u>				
SS	16.6	134.7	409.6	495.8
SI	10.0	109.6	250.7	311.4
IS	13.3	47.3	600.0	600.0
II	8.4	69.4	439.0	563.9

Shown are the median scores

amount of shock provided strong enough learning to obscure state-dependent effects. It is known that observation of state-dependency requires provision of a weak learning event (Overton, 1984). Our footshock training may have been too intense to allow the emergence of state-dependent effects. It should be noted, however, that the present results did not reflect ceiling effects.

Another possible problem in the present experiment was the chronic exposure to the glycemic states. The traditional state-dependent paradigm establishes the training and tests "states" only during the time of the learning and retention trials (Overton, 1984). Such a procedure allows the internal state to become a contextual cue associated specifically with the learning events. Longer exposure to insulin or to hyperglycemia in our procedures may have decreased the effectiveness of metabolic state in providing cues specific to the learning situation.

However, to the extent that we were modeling the situation as experienced by diabetic humans, they too, have chronic fluctuations in glycemic state whereby hyperglycemia and hypoglycemia are experienced at length and likely with some frequency as well. Thus, these altered glycemic states may not have a major impact upon learning.

Experiment 3b. Comparison of Retention for Passive Avoidance in Diabetic and Nondiabetic Rats After 1 Second or 2 Second Shocks

Methods

Subjects. Subjects for this experiment included the 26 rats from the diabetic and nondiabetic SS groups in Experiment 3a. They received training footshocks of 1-second duration, as previously described. Twelve additional diabetic rats and 12 additional nondiabetic rats were also included in the present manipulation. These rats (SS2) received training footshocks of 2-seconds duration. They were housed and maintained exactly as described in Experiment 3a, and given saline injections twice daily throughout the experiment. The additional 24 rats included here were divided among replications 2-4 as described in Experiment 3a, and trained and tested concurrently with the other groups of rats.

Apparatus. The apparatus was the same apparatus described in Experiment 3a.

Procedure. This study was conducted concurrently with Experiment 3a. The only additional detail for this experiment was the inclusion of the 2 additional groups (SS2), one composed of diabetic rats and the other of nondiabetic rats. In all other aspects, the experimental procedure for this experiment was identical to that of Experiment 3a. Plasma glucose concentrations and body weights of these groups were compared with ANOVAs along with

those of the other groups in Experiment 3a. Latencies and TTWs of the 4 groups in this experiment were compared using separate Kruskal-Wallis analysis by ranks, with differences among the individual groups determined with Mann Whitney U tests (Siegel, 1956).

Results and discussion

Plasma glucose concentrations and body weights of the rats in this experiment are shown in Tables 1 and 2 respectively. The 2 second shock groups (SS2) did not differ in blood glucose concentration or body weight from their 1 second shock counterparts (SS groups).

Behavioral results of Experiment 3b are shown in Figures 5-8. Latency data from pre-training and post-training sessions are shown in Figure 5. Figure 6 shows individual post-training latencies of rats in the 4 groups. There was a significant difference in post-training latencies among the groups as tested in the Kruskal-Wallis analysis ($H=16.16$, $P<0.01$). Mann Whitney U tests indicated that the diabetic (D) group given the 2-second shocks had a significantly higher median latency than all the other groups, while the D group given 1-second shocks had a significantly higher median latency score than its 1-second nondiabetic (N) counterpart. ($P_s<0.05$). The elevations noted in the N group given 2-second shocks relative to the N group given 1-second shocks approached significance ($P=0.06$). Pre-training latencies were significantly

Figure 5. Median latencies of nondiabetic (N) and diabetic (D) rats to enter the dark chamber of a shuttle box before (textured bars) and after (clear bars) footshock training.
*Significantly different from all other groups.
+Significantly different from the 1-second N group.

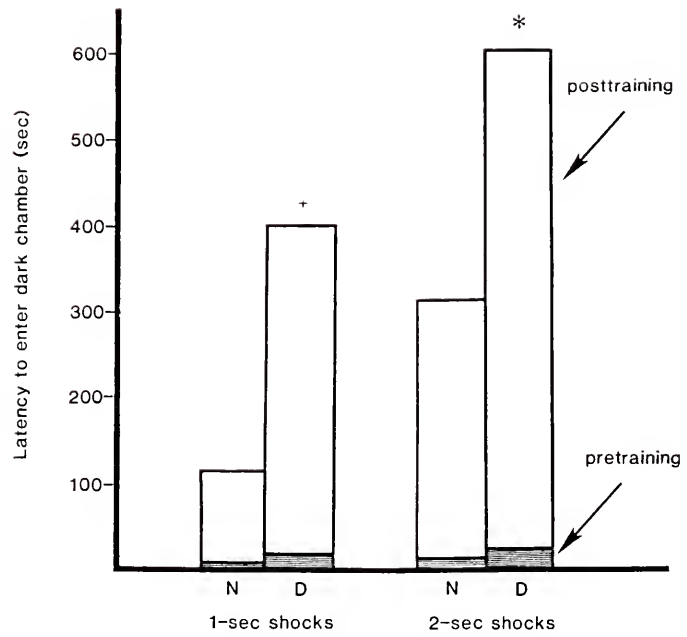


Figure 6. Median latencies of nondiabetic (N) and diabetic (D) rats to re-enter the dark chamber of a shuttle box where they had received footshock 10 days earlier with individual latencies indicated by solid circles.

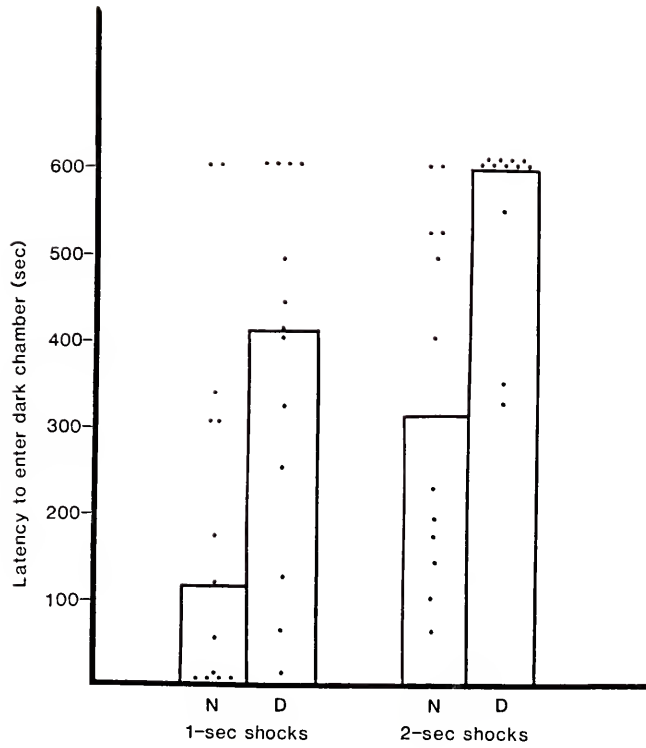


Figure 7. Median total time spent by nondiabetic (N) and diabetic (D) rats in the white chamber of a shuttle box before (textured bars) and after footshock training (clear bars). +Significantly different from the 1-second N group.

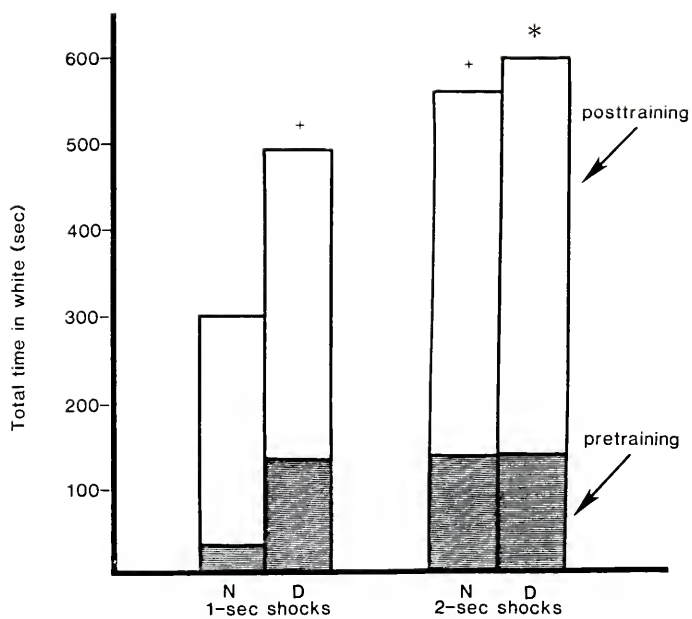
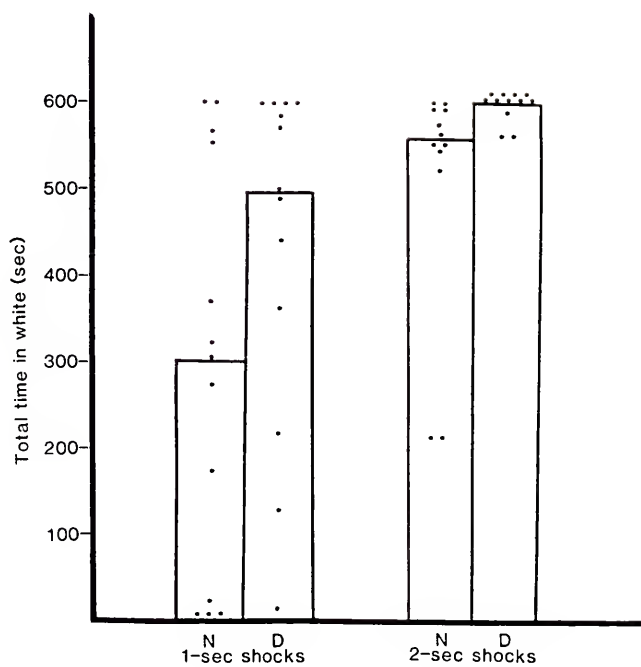


Figure 8. Median total time spent by nondiabetic (N) and diabetic (D) rats in the white chamber of a shuttle box during re-exposure to the box 10 days after receiving footshock in the dark chamber with individual scores indicated by solid circles.



different among the groups ($H=10.8$, $P<0.05$). However, all pretraining median latencies were less than 20 seconds. Moreover, when difference scores were calculated by subtracting pre-training latencies from post-training values and submitted to two-way ANOVA, diabetics showed significantly longer adjusted latencies than did nondiabetics at both shock durations (effect of diabetes, $F(1,49)=12.68$, $P<0.001$; effect of shock duration, $F(1,49)=8.75$, $P<0.005$).

There were also significant differences among groups in the total time spent in the white chamber (TTW) during the retention test session ($H=15.28$, $P<0.01$). Pretraining TTW scores were not significantly different ($H=3.02$, $P=0.404$). Median pre-training and post-training TTW scores are shown in Figure 7. Figure 8 shows individual post-training TTW scores. The D group given 2-second shocks spent significantly more time on white after training than did all other groups, and the N group given 2-second shocks had a higher TTW score than the N group given 1-second shocks ($P<0.05$). The difference between the two groups given 1-second shocks (N and D) did not reach significance ($P=0.09$). Adjusted TTW scores (post-training scores minus pre-training scores) indicated an effect of diabetes which just missed the usual accepted alpha level of 0.05 ($F(1,49)=3.77$, $P=0.0583$). There was a significant effect of shock duration on these adjusted TTW scores ($F(1,49)=12.80$, $P<0.001$).

These data demonstrated better retention for the passive avoidance task than in diabetic rats than in nondiabetic rats. Thus, while all the rats entered the dark chamber with very short latencies prior to footshock training, and spent little time in the white chamber, the four training footshocks produced significantly longer latencies to re-enter the dark chamber in diabetics than in nondiabetics. Moreover, the diabetic rats spent significantly more time in the white chamber than did the nondiabetics, despite the initial preference for the dark chamber by all the rats.

These retention differences may be related to known biochemical differences in diabetic and nondiabetic rats. For example, diabetics have been shown to have elevated resting corticosterone concentrations, as well as higher peak increments following stress (L'Age et al., 1974; Tornello et al., 1981b). Norepinephrine turnover is reduced in interscapular brown adipose tissue, heart and pancreas of streptozotocin diabetic rats (Yoshida et al., 1985), while concentrations of DBH and norepinephrine in plasma are elevated (Berkowitz et al., 1980). Thus, diabetic rats may enter a learning situation in a very different basal hormonal state than nondiabetic rats. Such differences are known to influence subsequent responses to the training episode (Leshner, 1975).

Differential hormonal states have been demonstrated to be relevant to learning. It was shown that artificially

elevating post-training plasma hormone concentrations by administration of either ACTH or epinephrine facilitated retention for weak footshock training (Gold & McGaugh, 1978). Post-training administration of higher doses of ACTH or epinephrine actually impaired retention. These treatments were compared to the effect of employing stronger footshock in producing better retention (Gold & van Buskirk, 1976). Similarly, enhancement of learning due to increases in total footshock delivered might be accompanied by higher corticosterone concentrations.

Corticosterone concentrations have been found to be responsive to both increased shock duration (Friedman, Ader, Grotta, & Larson, 1967) and increased shock intensity (Keim & Sigg, 1976). In the present experiment, diabetics showed better retention than nondiabetics following both 1-second and 2-second shocks. One possible factor in this superiority could have been that diabetics experienced larger corticosterone increments in response to footshock than did nondiabetics.

In order to evaluate the effects of our footshock upon circulating hormone levels, plasma corticosterone concentrations following training and testing for passive avoidance were determined in Experiment 4.

Experiment 4. Plasma Corticosterone Concentrations
Following Footshock Training and Retention Testing
in Diabetic and Nondiabetic Rats

A number of experimental stressors cause the release of ACTH, which in turn stimulates the adrenal cortex to increase synthesis and release of corticosteroids. Elevations of plasma corticosterone concentrations have been reported following cold, immobilization and footshock stress in rats (Keim & Sigg, 1976; Lenox, Kant, Sessions, Pennington, Mougey, & Meyerhoff, 1980; Odio & Maickel, 1985). ACTH concentration in plasma increased in response to both footshock training and the re-exposure to the fear cues during retention testing (van Wimersma Greidanus et al., 1977). The hormonal response was much larger when rats were replaced in the training situation than when they were given footshock. Thus, there may be even greater sensitivity of the hypothalamo-pituitary-adrenal system to psychological stress (e.g. fear) than there is to some physiological stresses (e.g. footshock).

This hormonal sequence of ACTH release followed by corticosterone release has been associated with the storage of memory. Some evidence has been reported which indicates that the concentration of ACTH in plasma following training is a determinant of the strength of the memory (Gold & van Buskirk, 1976). In Experiment 3b, diabetics exhibited better retention for passive avoidance training than nondiabetics with both 1-second and 2-second duration

shocks. One possible explanation for this superiority would be that diabetics had higher baseline corticosterone concentrations (Tornello et al., 1981b) and/or experienced greater corticosterone increments in response to footshock stress.

To investigate these possibilities, corticosterone concentrations were measured in plasma samples obtained 15 minutes after footshock training and 15 minutes after re-exposure to the shuttle box during retention testing. The 15 minute timepoint was chosen because it was reported to be the approximate time of maximal elevation of corticosterone concentrations following footshock in some previous studies (e.g., Madden, Rollins, Anderson, Conner, & Levine, 1971). In addition this timepoint corresponds to the time when neurochemical changes in response to footshock training (Tanaka et al., 1982) and retention testing (Herman et al., 1982) have been found. It was hoped that both neurochemical and peripheral hormonal events linked to the training and testing situations could be identified and thus a timepoint most likely to accommodate all the various parameters was selected.

Methods

The rats and behavioral procedures were as in Experiments 3a and b. Blood samples were collected following footshock training by tail nick, as described in Experiment 1. Blood samples were collected following

retention testing by decapitation as described in Experiment 3. The samples were centrifuged and the plasma separated and stored at -70°C . Plasma was also collected from 7 diabetic and 7 nondiabetic rats within 3 minutes of removal from their home cages, in order to establish baseline corticosterone concentrations. Baseline plasma samples were collected between 1:00 P.M. and 3:00 P.M., approximately the same time at which training and testing were conducted.

Corticosterone concentrations were measured using the competitive protein-binding assay of Murphy (1967). Briefly, 10 μl samples of plasma (40 μl of plasma from unstimulated rats) were extracted in 2 mls of methanol, and duplicate 500 μl aliquots of the extract were dried under a warm air stream along with corticosterone standards (.25, 25, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 ng). After drying was complete (approximately 20 minutes), 1 ml of a reaction mixture containing dog plasma (from a dexamethasone-treated dog) and [^3H]-corticosterone was added to each tube. The samples and standards were then incubated for 5 minutes at $40-45^{\circ}\text{C}$ and then placed on ice for 20 minutes to bring the reaction to equilibrium. Dextran-coated charcoal (0.2 ml) was then added and 10 minutes later the tubes were centrifuged for 10 minutes at 4°C . The supernatant was decanted into scintillation vials and Beta activity was counted.

The standards were fitted to a straight line using the log-logit transformed data, and samples were evaluated by comparison with the standard values along the linear regression line. Group means were compared using two-way ANOVA.

Results and Discussion

The mean corticosterone concentrations of the various groups are shown in Figures 9, 10 and 11. All values measured 15 minutes after training and testing were elevated relative to the afternoon baseline concentrations, although the baseline measures were not compared statistically with the learning corticosterone measures, since they were not a part of the statistical design of the learning paradigms. The mean baseline concentrations of diabetic and nondiabetic rats were compared and were found to be indistinguishable statistically. Among the diabetic state dependent groups (Figure 9), the only significant effect was the effect of session ($F(1,35)=8.94$, $P<0.01$), with test values being higher than training values. Among nondiabetic state dependent groups (Figure 10), there was an interactive effect of treatment with session ($F(1,34)=4.18$, $P<0.02$). Thus, values were generally higher after testing than after training, but in addition, there were differential changes between the sessions. In particular, the SI group had very high corticosterone concentrations after testing, suggesting an interaction of hypoglycemia and psychological fear. At

Figure 9. Mean plasma corticosterone concentrations of diabetic state-dependent groups following footshock (Training) and re-exposure to fear cues (Testing). SS=saline prior to training and testing, SI=saline prior to training and insulin prior to testing, IS=insulin prior to training and saline prior to testing, II=insulin prior to training and testing, Basal=non-stressed controls. *Testing concentrations significantly higher than Training concentrations.

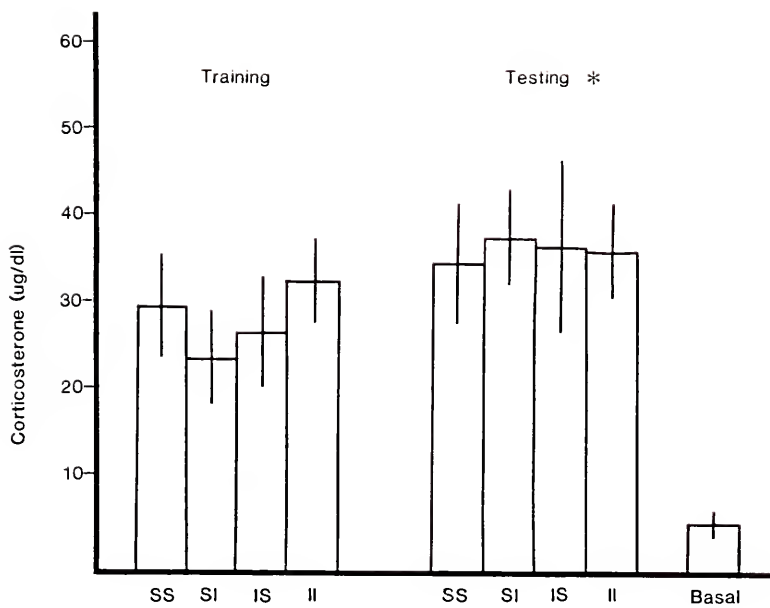


Figure 10. Mean plasma corticosterone concentrations of nondiabetic state-dependent groups following footshock (Training) and re-exposure to fear cues (Testing). SS=saline prior to training and testing, SI=saline prior to training and insulin prior to testing, IS=insulin prior to training and saline prior to testing, SSTP=saline prior to training and testing and streptozotocin administration following training, Basal=non-stressed controls. *Testing concentrations significantly higher than training concentrations. +Significantly different from all other Testing groups.

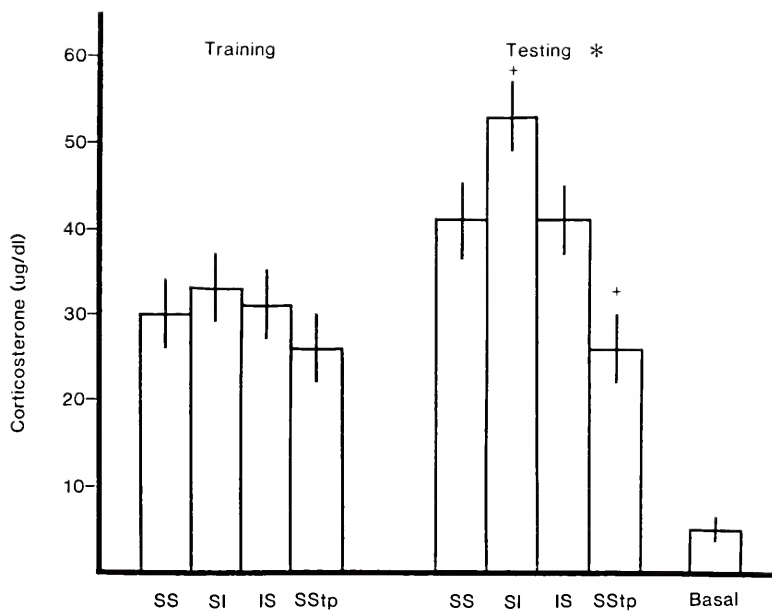
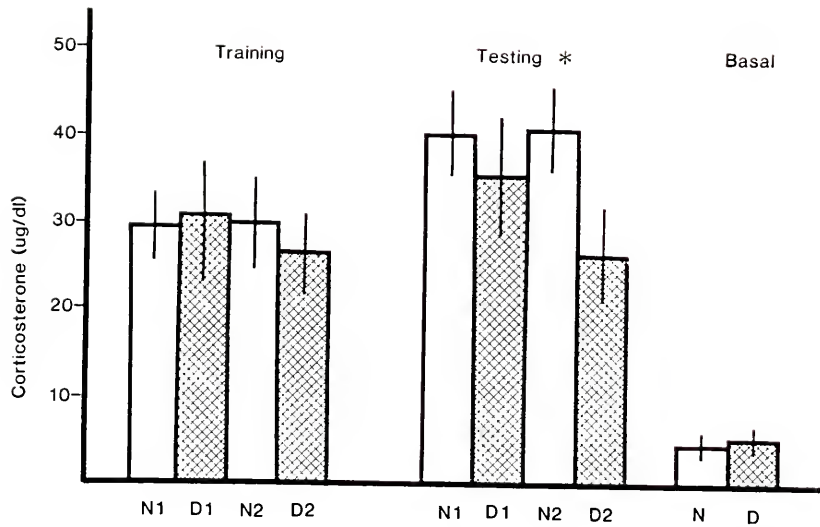


Figure 11. Mean plasma corticosterone concentrations of saline-treated nondiabetic and diabetic rats after 1-second (N1 and D1) or 2-second footshock (N2 and D2) after footshock training and after retention testing and of groups (N and D) not exposed to any stress (Basal). *Testing concentrations significantly higher than Training concentrations.



the other extreme were the SSTP rats which showed essentially no difference in corticosterone response to testing than to training.

Among the fear cue re-exposure groups (Figure 11), there was a significant main effect of session ($F(1,33)=5.34$, $P<0.05$), with concentrations at testing, again, higher than those at training.

The present results confirm the previous finding of greater pituitary-adrenal activation following retention testing than following footshock training (van Wimersma Greidanus et al., 1977). However, the differences in corticosterone values found here failed to provide any hormonal basis for the superiority of diabetics in retention for the avoidance task. It is possible that different ACTH elevations occurred in the diabetics and nondiabetics despite similar corticosterone increments (De Nicola et al., 1977). Another possibility is that the timecourse of the hormonal response to stressful stimuli is different in diabetic and nondiabetic rats. Thus, maximal responses to stress in diabetic and nondiabetic rats may be indistinguishable while duration of the increments are longer-lasting in diabetics. Such alterations in timecourse have been noted in aging rats (Sapolsky, Krey, & McEwen, 1983). In addition, metabolic disturbances associated with epinephrine or cortisol infusion endure much longer in diabetics than in nondiabetics (Shamoon et al., 1980).

Finally, 15 minutes post-stress may not be the optimal time for the measurement of peak elevations of corticosterone in response to stress. There have been studies which have reported maxima at 30 to 60 minutes (Bassett & Cairncross, 1975; Keim & Sigg, 1976; Odio & Maickel, 1985).

Experiment 5. Twenty-Four Hour Catecholamine Excretion
in Diabetic and Nondiabetic Rats

There have been reports of decreased norepinephrine turnover in some peripheral tissues (Yoshida et al., 1985) and decreased plasma epinephrine and norepinephrine (Berkowitz & Head, 1978) in diabetic rats. However, DBH, an important synthetic enzyme is elevated (Schmidt et al., 1981), as is norepinephrine for at least the first two months following streptozotocin administration, and thus the diabetic may be either advantaged or disadvantaged in the face of stress, depending upon whether existing plasma catecholamine concentrations or synthetic capability is more critical. In any case, plasma catecholamine responses to footshock have been shown to be relevant to later demonstration of good retention of passive avoidance training (McCarty & Gold, 1981). To extend these previous findings regarding peripheral catecholamine status in the diabetic rat and its possible relationship to learning an aversive task, the present experiment was conducted in which 24-hour excretion of catecholamines was measured in diabetic and nondiabetic rats both under basal conditions and during

the 24 hours immediately following footshock training. Different baseline catecholamine turnover might have facilitated retention for the passive avoidance task in the diabetic rats. Furthermore, some evidence exists suggesting that the stress response in diabetics may be more prolonged (Shamoon et al., 1980). Prolonged plasma catecholamine elevation following footshock might facilitate memory storage and, in addition, might be reflected in greater 24-hour urine concentrations. These possibilities were investigated in this experiment.

While it would have been preferable, perhaps, to measure plasma catecholamines directly following the training and testing procedures, this was deemed an impossible procedure to design into the present studies. However, measurement of urinary excretion of catecholamines has the advantage of being noninvasive and producing no disturbance of the rats during the measurement period. In addition, urinary epinephrine and norepinephrine are thought to reflect general peripheral activity of these hormones (Overy, Pfister, & Chidsey, 1967; Roy et al., 1977), and, of course, peripheral catecholamine activity has been correlated with learning (Gold & Delanoy, 1981).

One potential problem in comparing urinary catecholamines in diabetic and nondiabetic rats is that of altered renal function occurring in diabetes. Glomerular filtration rate (GFR) was reduced in the alloxan-diabetic

rat one month after induction of diabetes (Michels, Davidman, & Keane, 1981). However, GFR was elevated for at least 8 days following administration of streptozotocin (Carney, Wong, & Dirks, 1979). Elevated GFR in streptozotocin-diabetic rats was noted only in those diabetic rats receiving small daily doses of insulin (Hostetter, Troy, & Brenner, 1981). Diabetic rats not receiving insulin had reduced GFR relative to nondiabetic rats.

Since catecholamine excretion would appear to be a constant fraction of GFR (Overy et al., 1967), alterations in GFR could lead to spurious differences in measured catecholamine excretion between diabetic and nondiabetic rats. To determine whether this would be a problem in the present study, GFR was estimated by creatinine clearance.

Methods

Animals and housing

Twenty-four hour urine samples were collected from 9 diabetic and 11 nondiabetic rats from replication 4 of the learning experiment (protocol 3b). All housing and maintenance details were therefore identical, except that during urine collection, the rats were placed on a special rack in their home cages.

Catecholamine determinations

Urine was collected in beakers containing 0.5 ml of 3N hydrochloric acid (HCL). At the end of the 24-hour

collection period, urine volume was measured and urine was frozen at -70°C until biochemical assays were performed.

Samples (5 ml) were placed into beakers, and 15 ml of 0.1M phosphate buffer (pH 7.0) containing 40 ng of dihydroxybenzylamine (DHBA, Sigma) was added. Contents of the beaker were poured over cation exchange columns (Bio Rex 70, BioRad Laboratories, Richmond, CA) and washed with 10 ml of distilled water (DH_2O). The cation exchange columns were acidified with 1.3 ml of 0.7M sulfuric acid, and the catecholamines were eluted from the column with 4 ml of 2M $(\text{NH}_4)_2\text{SO}_4$. The eluent was collected into vials containing 50 mg of acid-treated alumina, 500 μl of Tris buffer (pH 8.6) was added, and the samples were agitated for 15 minutes. The samples were washed once with DH_2O , and an alumina slurry was loaded onto microfilters and centrifuged to dryness. The catecholamines were eluted from the dried alumina with 200 μl of 0.1N perchloric acid and these fractions were submitted to high performance liquid chromatography (HPLC).

The HPLC system (BAS LC-304) consisted of a refrigerated autosampler, C18 column (Bondapak, 5 μm), glassy carbon electrode with an applied voltage of +0.65Volts, and a Hewlett Packard integrator. The mobile phase contained 91% 0.1M monochloroacetate buffer (pH 3.0) with 2mM Na_2EDTA and 600 mg/L sodium octyl sulfate : 9% acetonitrile (v/v). The flowrate was 1.5 ml/minute. Catecholamine concentrations

were determined automatically by comparison with external standards. Calculated values included corrections for recovery of DHBA (approximately 45%) added to the samples prior to the extraction procedure and adjusted for concentration of the samples.

The urine catecholamine concentrations of diabetic and nondiabetic groups, both baseline and after footshock, were compared statistically by two-way ANOVA.

Determination of glomerular filtration rate

Both urine creatinine excretion and plasma creatinine concentration were determined using the Jaffe colorimetric reaction (Heinegard & Tiderstrom, 1973). Briefly, this procedure (Sigma; kit #555A) involved addition of alkaline picrate, which reacts with creatinine to form a complex that absorbs light at 500nm. After reading absorbance with a spectrophotometer (Bausch & Lomb), acid was added to destroy all creatinine-related absorbance, and a second reading measured contaminants included in the first reading. The second reading was subtracted from the first and divided by absorbance of a creatinine standard (1 mg/dl) equal in volume to sample aliquots. Calibration curves assured the linearity of the assay within the range measured.

Twenty-four hour urinary excretion of creatinine (UV_{Cr}) was calculated by multiplying urinary volume (UV) by urine creatinine concentration. GFR was estimated by dividing UV_{Cr} by plasma creatinine concentration.

Urine creatinine concentration, plasma creatinine concentration, and GFR values and were compared with Student's t-tests or two-way ANOVA.

Results and Discussion

Plasma creatinine concentrations, total creatinine excretion and GFR are shown in Table 4. Statistical analysis indicated that there were no differences in GFR among the two groups ($P > 0.05$). However, both plasma creatinine concentration ($t = -8.36$, $P < 0.05$) and urinary creatinine excretion ($t = -8.79$, $P < 0.05$) of diabetics were significantly higher than those of nondiabetics.

Excretion of catecholamines is frequently normalized to body weight or to creatinine excretion. The body weight normalization was problematic in the present experiment, given the large systematic disparity in body weight between diabetic and nondiabetic rat (Mean = 403 ± 10 in diabetic rats vs 522 ± 3 in nondiabetic rats), which could inflate the estimation of urinary catecholamine excretion in the diabetics. Since urinary creatinine excretion was elevated in diabetics, the normalization of catecholamine excretion to this value would provide a conservative estimate of reported increases in diabetics, although, it would overstate reduced values. Because both normalization procedures might be subject to error, the data were calculated and analyzed with both normalizations, and in addition, total excretion, with no normalization, was

TABLE 4

Renal and Metabolic Variables Associated
with Footshock in Diabetic and Nondiabetic Rats

<u>Group</u>	<u>Variable</u>	<u>Session</u>	
		Baseline	Footshock
<u>Nondiabetics</u>			
	Bwt	522 $\pm$ 4	527 $\pm$ 5
	P _{Cr}	-	0.55 $\pm$ 0.04
	UV	22.4 $\pm$ 3.2	23.0 $\pm$ 1.3
	UV _{Cr}	18.0 $\pm$ 0.8	18.9 $\pm$ 0.5
	GFR	-	2.26 $\pm$ 0.24
<u>Diabetics</u>			
	Bwt	403 $\pm$ 10	384 $\pm$ 10
	P _{Cr}	-	1.30 $\pm$ 0.26
	UV	293 $\pm$ 22	263 $\pm$ 13
	UV _{Cr}	28.4 $\pm$ 1.0	27.6 $\pm$ 1.0
	GFR	-	2.11 $\pm$ 0.66

Bwt, body weight (g)

P_{Cr}, plasma creatinine concentration (mg/dl)

UV, 24 hour urinary volume (ml)

UV_{Cr}, 24 hour urinary creatinine excretion (mg)

GFR, glomerular filtration rate (ml/min)

evaluated. Figures 12, 13 and 14 present the urine catecholamine data normalized to body weight, normalized to creatinine, and with no normalization, respectively. Epinephrine excretion was found to be significantly lower in diabetic than in nondiabetic rats only with the creatinine normalization $F(1,18)=16.8$, $P<0.001$). However, with all three calculations, there were significant interactions of condition with session (baseline vs footshock; Body weight-- $F(1,18)=6.85$, $P<0.02$; Creatinine-- $F(1,18)=5.3$, $P<0.04$; Total excretion-- $F(1,18)=4.5$, $P<0.05$); diabetics had higher epinephrine excretion following footshock than during baseline measurement, while nondiabetics had lower epinephrine excretion following footshock than during the baseline period.

Norepinephrine excretion was significantly greater in diabetics than in nondiabetics by all three calculations (Body weight-- $F(1,18)=39.25$, $P<0.001$; Creatinine-- $F(1,18)=4.77$, $P<0.05$; Total excretion-- $F(1,18)=13.45$, $P<0.01$).

Dopamine excretion normalized to body weight was greater in diabetics than in nondiabetics ($F(1,18)=26.68$, $P<0.001$). While excretion normalized to creatinine was not different in diabetics and nondiabetics, total excretion was also greater in diabetics ($F(1,18)=4.62$, $P<0.05$).

The data demonstrated reduced urinary epinephrine excretion in diabetic rats under basal conditions but

Figure 12. Urinary excretion of epinephrine, norepinephrine and dopamine (ug excreted per Kg body weight) during a 24 hour baseline measure (Bas) and during the 24 hours following footshock (FS) in nondiabetic and diabetic rats. *Diabetics significantly different from nondiabetis. +Significant interactive effects of condition (diabetics vs nondiabetics) and session (Bas vs FS).

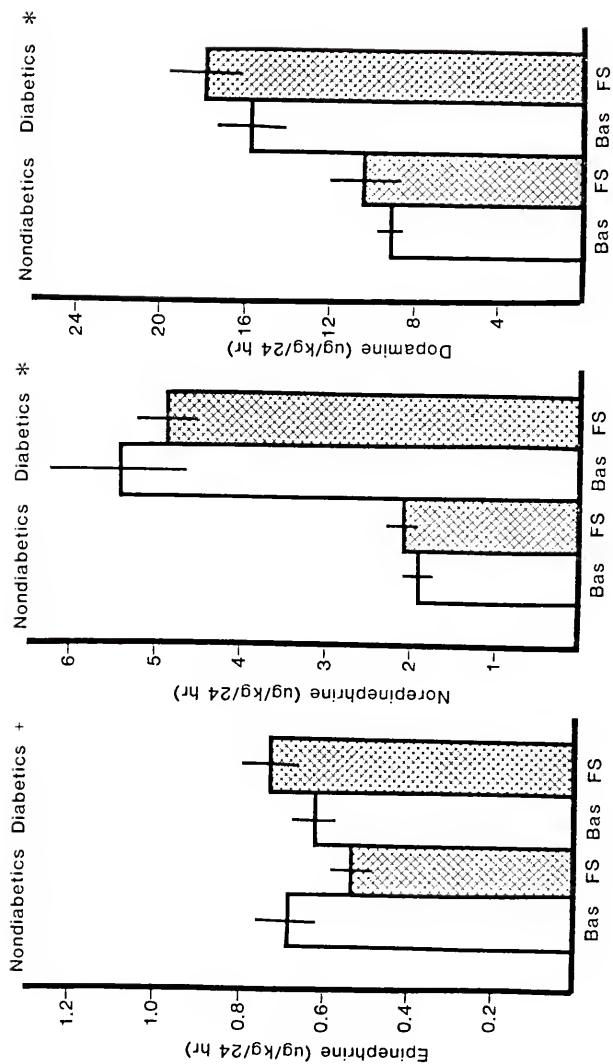


Figure 13. Urinary excretion of epinephrine, norepinephrine and dopamine (ng/mg creatinine) during a 24 hour baseline measure Bas) and during the 24 hours following footshock (FS) in nondiabetic and diabetic rats. *Diabetics significantly different from nondiabetics. +Significant interactive effects of condition (diabetics vs nondiabetics) and session (Bas vs FS).

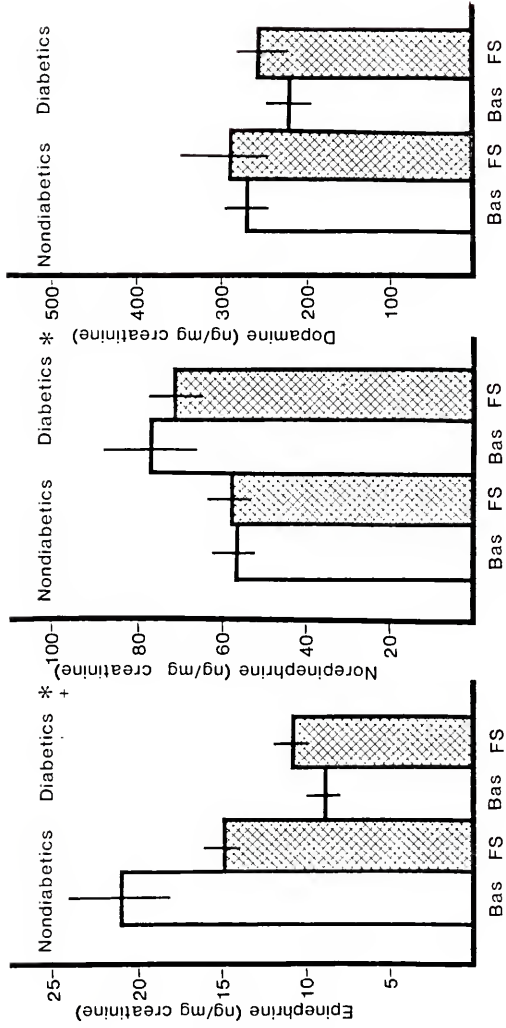
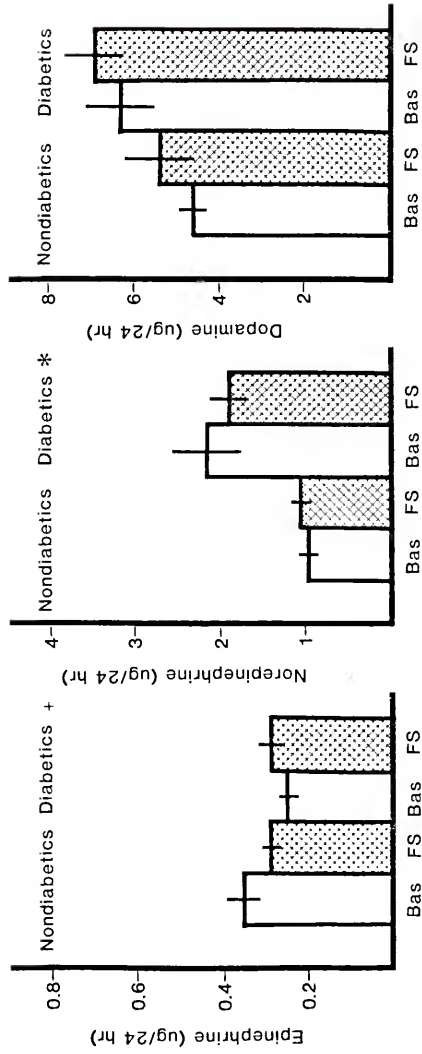


Figure 14. Urinary excretion of epinephrine, norepinephrine and dopamine (total ug excreted per 24 hours) during a 24 hour baseline measurement (Bas) and during the 24 hours following footshock (FS) in nondiabetic and nondiabetic rats. *Diabetics significantly different from nondiabetics. +Significant interactive effect of condition (diabetics vs nondiabetics) and session (Bas vs FS).



elevations in excretion following footshock stress, in contrast with reduced excretion in nondiabetic rats. Urinary norepinephrine excretion was consistently elevated in diabetic rats. Dopamine excretion appeared elevated in diabetic rats in 2 of the 3 calculations. It has been suggested that epinephrine excretion is a reflection of adrenomedullary activity (Overly et al. 1967), while norepinephrine excretion reflects sympathetic neuronal outflow (Roy et al., 1977). In contrast to circulating plasma as the source of urinary epinephrine and norepinephrine, dopamine excretion reflects primarily local production in the kidney (Lee, 1982).

The elevation in urinary norepinephrine excretion in diabetics in the present experiment thus suggest an increase in sympathetic activity in these rats. It cannot be determined from these data whether synthesis is increased or metabolism decreased. However, this result is in agreement with the finding of elevated concentrations of DBH and norepinephrine in plasma of the streptozotocin-diabetic rat (Berkowitz et al., 1980). Moreover, since turnover of norepinephrine in peripheral tissues was reduced in diabetic rats (Yoshida et al., 1985), alterations in clearance may be the source of elevated excretion. The differential urinary excretion of epinephrine in diabetic and nondiabetic rats suggest modifications of adrenomedullary response in the diabetic rats as well. These changes may have been involved

in the retention differences between diabetic and nondiabetic rats, given that epinephrine responses to footshock have been shown to correlate with later retention for passive avoidance (McCarty & Gold, 1981).

The increase in dopamine excretion in diabetic rats, suggesting increases in renal production of dopamine (Lee, 1982) may be related to changes in urine volume and electrolyte excretion in the diabetic rat (Carney et al., 1979) or to structural changes, in particular glomerular hypertrophy, which occur in diabetes (Seyer-Hansen, Hansen, & Gundersen, 1980). While this change in dopamine excretion is probably not related directly to the behavioral differences, as are alterations in epinephrine and norepinephrine excretion, it does demonstrate the extent of the hormonal disruption which occurs in diabetes.

Experiment 6. Neurochemical Correlates of Aversive Learning in Diabetic and Nondiabetic Rats

As detailed in the introduction, a number of changes in the concentrations of monoamines and their metabolites in the CNS have been associated with both footshock training and with retention of an aversive task. Norepinephrine seems particularly relevant (e.g., Gold & Murphy, 1980). Given that footshock seems to be accompanied by decreases in norepinephrine in forebrain (possibly indicating increased turnover), it is noteworthy that diabetic rats have been reported to have higher NE concentrations than nondiabetic rats in whole brain (Trulson & Himmel, 1985), and in several

specific regions, including thalamus, hypothalamus, medulla and midbrain (Bitar et al., 1986). There has been no speculation as to whether these elevations would be advantageous or disadvantageous in a stressful situation.

This experiment evaluated catecholamine concentrations in several brain regions, under basal conditions, after footshock and after re-exposure of rats to the fear cues following 1-second or 2-second training footshocks.

Methods

Animals and housing

Several groups of rats were included in these neurochemical evaluations. All had been injected with either streptozotocin or vehicle and were housed and maintained exactly as described in the previous experiments. Groups from Experiment 3b comprised the fear cue re-exposure groups. In addition, 8 diabetic and 8 nondiabetic rats not involved in passive avoidance training were taken directly from their cages to be sacrificed. These rats provided baseline neurochemical values for comparison with those of rats re-exposed to fear cues after 1- or 2-second shocks. Another two groups of rats (6 diabetics and 5 nondiabetics) were sacrificed 10 minutes after receiving a 3-second 1mA footshock. This last group was not included in the statistical comparison with the other groups, but metabolite data from these rats were included for qualitative comparison of responses to footshock, a physiological

stressor, with responses to fear upon re-exposure to the shuttle box.

Tissue preparation

As described in experiment 3b, the animals were decapitated 15 minutes after fear-cue reexposure. Their brains were quickly removed and placed onto an ice cold petri dish. The following brain regions were dissected and frozen at -70°C until biochemical analyses were conducted: Frontal cortex (Mean weight $\pm$ standard error-- 89.2 ± 3.8 mg) included the frontal poles removed by a coronal cut just rostral to the olfactory tubercles after removal of the olfactory bulbs. Hypothalamus (81.0 ± 3.0 mg) was removed with a curved forceps by making cuts rostrally at the level of the optic chiasm, caudally at the level of the mamillary bodies and laterally along the hypothalamic sulci and lifting a 3 mm deep section. Amygdala and the overlying pyriform cortex (77.9 ± 2.2 mg) lying just lateral to the hypothalamus were next excised and removed using a curved forceps. The brain was next turned over exposing the dorsal surface and the cortex was peeled back. Then the hippocampus (188.6 ± 5.1 mg) was teased away from the septum rostrally and lifted away from the underlying tissue. At the caudal border, a forceps was used to separate the hippocampus from the posterior cortex. Finally brainstem (234.7 ± 3.6 mg) was separated by cuts at the obex caudally

and the the aqueduct rostrally with a straight-edge razor and the dura was removed.

Tissues were homogenized in .2N perchloric acid buffer containing EDTA (10^{-5} M) and 1uM isoproterenol (1:10 w/v), then centrifuged at 13,000 rpm for 10 minutes. The supernatant was decanted into vials for HPLC determinations.

The HPLC system consisted of a Waters isocratic pump, a Kontron autosampler, a reverse phase C8 column, electrochemical detector (BAS 4A) with a glassy carbon working electrode with applied voltage of +0.7 volts. This was connected to a Perkin Elmer Sigma 15 data acquisition module which automatically integrated the peak areas comparing those of unknowns with internal and external standards for determination of actual concentrations. The mobile phase (pH 3.35) consisted of 0.1M Na_2HPO_4 , 14% methanol, 1.6 g/l sodium octyl sulfate and 10uM triethylamine. Flow rate was 1.0 ml/minute.

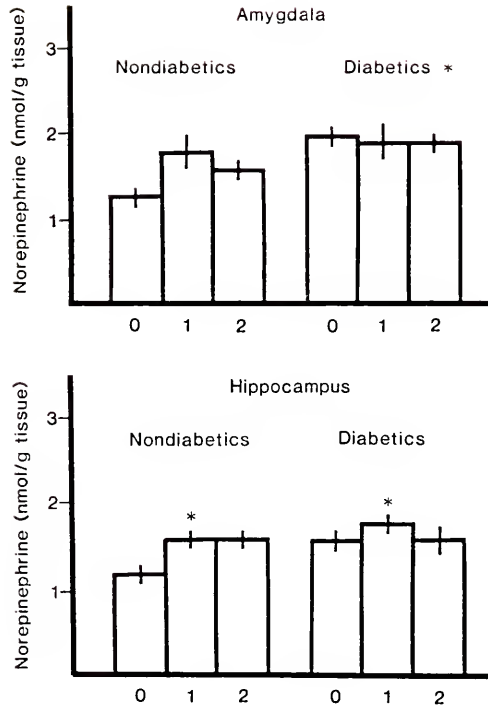
Data were analyzed statistically using two-way ANOVA and Duncan's post hoc tests.

Results and Discussion

A number of significant differences were found among the groups. In the amygdala (Figure 15, upper panel), diabetic had significantly higher concentrations of NE than did nondiabetics ($F(1,61)=11.04$, $P<0.01$). In the hippocampus (Figure 15, lower panel), there were differences in NE among the different shock duration groups. Both 1-second shock

Figure 15. Upper panel: Norepinephrine concentrations in amygdala of nondiabetic and diabetic rats sacrificed 15 minutes after re-exposure to fear cues.. 0=no exposure controls, 1=1-second footshock, 2=2-second footshock. *diabetics significantly different from nondiabetics.

Lower panel: Norepinephrine concentrations in hippocampus. *Significantly different from no exposure controls.



groups had significantly more NE than their respective no shock groups, while the diabetic 2-second shock group was not different from its control ($F(2,63)=3.44$, $P<0.05$).

Several differences occurred in frontal cortex (Figure 16). First, DA (upper panel) was significantly elevated in the 2-second shock groups ($F(2,59)=6.44$, $P<0.01$). DOPAC was also higher in these groups ($F(2,42)=17.58$, $P<0.001$). In addition to the effects of retention testing, DOPAC concentrations were also significantly lower in the diabetic groups than in the nondiabetic groups ($F(1,42)=6.77$, $P<0.02$). DOPAC to DA ratios were significantly lower in diabetics than in nondiabetics ($F(1,38)=11.04$, $P<0.01$) as well. Although not analyzed along with the shock duration retention group data, footshock groups had even higher dopamine concentrations than the groups sacrificed after retention testing. Following footshock, diabetics had significantly more dopamine than nondiabetics ($F(1,15)=6.47$, $P<0.05$).

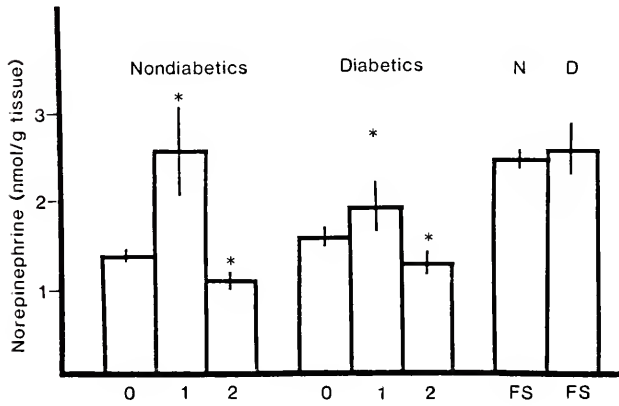
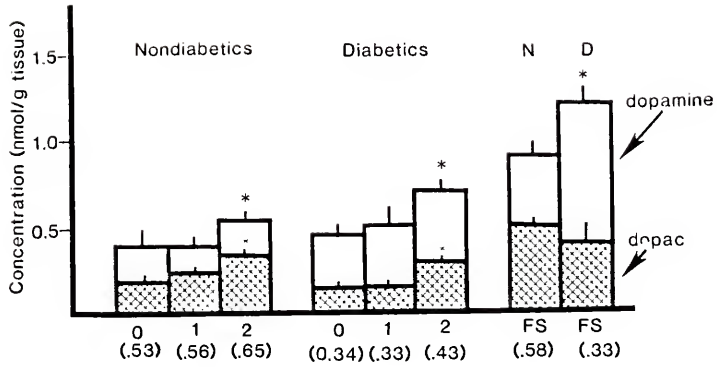
The bottom panel of Figure 16 shows NE concentrations in frontal cortex. Rats tested after 1-second shocks had higher NE concentrations than control rats, while the 2-second shock groups did not differ significantly from control rats ($F(2,64)=6.68$, $P<0.01$). Footshock produced very high NE concentrations, although the diabetic and nondiabetic footshock groups were not statistically different from one another.

Figure 16. Upper panel: Dopamine (clear bars) and DOPAC (textured bars) concentrations in frontal cortex of nondiabetic and diabetic rats sacrificed 15 minutes after re-exposure to fear cues. 0=no exposure controls, 1=1-second footshock, 2=2-second footshock and of rats sacrificed 10 minutes after receiving a single 3-second 1mA footshock (FS). DOPAC to dopamine ratios shown in parentheses. *2-second shock groups significantly different from no exposure controls; FS diabetics significantly different from FS nondiabetics for DOPAC, dopamine and ratio.

Lower panel: Norepinephrine concentrations.

*Significantly different from no exposure controls.

Frontal Cortex



In hypothalamus (Figure 17), both DA (upper panel) and NE (lower panel) concentrations were higher in diabetics than in nondiabetics ($F(1,65)=6.37$ and 11.52 respectively, $P<0.02$). Concentrations of both DA and NE were significantly reduced in both the 1-second and 2-second shock groups ($F(2,65)=15.09$ and 31.44 respectively, $P<0.001$), and 1-second shock groups had significantly lower concentrations than did 2-second shock groups. DOPAC concentrations differed significantly among the fear cue reexposure groups ($F(2,60)=3.51$, $P<0.001$), with 2-second shock groups having significantly lower concentrations than the no shock controls, while the 1-second groups had intermediate concentrations not differing from either 2-second groups or no shock controls. There significant differences in the ratio of DOPAC to DA ($F(2,60)=4.17$, $P<0.05$), with the 1-second groups elevated, and the 2-second groups reduced, relative to no shock controls. The two shock retention groups thus differed significantly from each other but neither differed significantly from the no shock control group. Diabetics had significantly lower DOPAC to DA ratios than nondiabetics ($F(1,60)=9.93$, $P<0.001$). Particularly interesting in hypothalamus were the very large DOPAC to DA ratios following footshock in both diabetic and nondiabetic groups.

Brainstem NE (Figure 18) was also higher in diabetics than in nondiabetics ($F(1,64)=19.15$, $P<0.001$). Moreover,

Figure 17. Upper panel: Dopamine (clear bars) and DOPAC (textured bars) concentrations in hypothalamus of nondiabetic and diabetic rats sacrificed 15 minutes after re-exposure to fear cues and of rats sacrificed 10 minutes after receiving a single 3-second 1mA footshock (FS). 0=no exposure controls, 1=1-second footshock, 2=2-second footshock. +Diabetics significantly different from nondiabetics. *Significantly different from no exposure controls.

Lower panel: Norepinephrine concentrations. +Diabetics significantly different from nondiabetics. *Significantly different from no exposure controls.

Hypothalamus

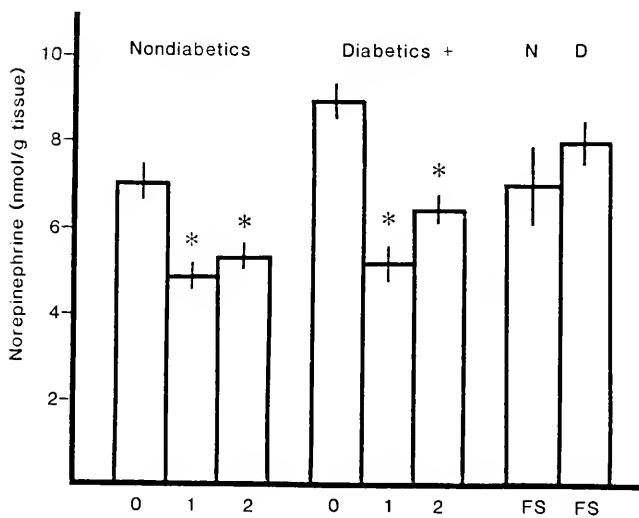
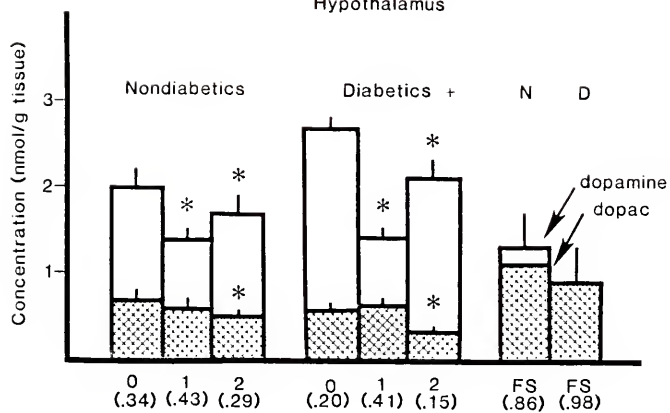
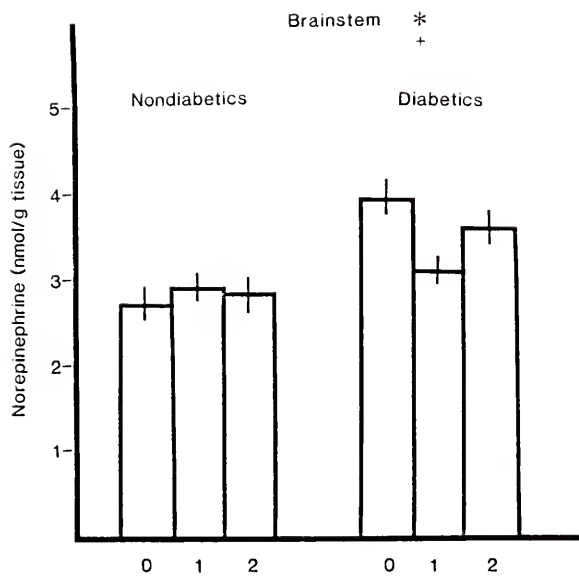


Figure 18. Norepinephrine concentrations in brainstem of nondiabetic and diabetic rats sacrificed 15 minutes after re-exposure fear cues. 0=no exposure controls, 1=1-second footshock, 2=2-second footshock. *Diabetics significantly different from nondiabetics. +Interactive effect of condition (diabetics vs nondiabetics) and shock duration(0, 1 or 2).



there was an interactive effect of condition and shock($F(2,64)=4.67, P<0.02$), whereby in the diabetics the 1-second group was significantly reduced relative to no shock controls and the 2-second group was intermediate, while the three nondiabetic groups were virtually indistinguishable.

Our data are consistent with a number of previous reports concerning the responses of central catecholamines to footshock or to re-exposure to an environment where footshock was previously administered. For example, we found elevations in DOPAC concentrations in frontal cortex in both 1-second and 2-second groups following retention testing (Herman et al., 1982). However, there were not significant elevations in the DOPAC:DA ratios in the present study, as have been reported previously (Dunn, Elfvin, & Berridge, in press), either following footshock training or following re-exposure to fear cues. We also found elevations in DA in re-exposure groups.

Such increases in concentration of the parent amine are not usually found, possibly because DA is rapidly resynthesized (Bliss, Ailion, & Zwanziger, 1968). However, increases in dopamine concentration following footshock stress have been found when animals were pretreated with α -methyl-para-tyrosine, which prevents synthesis of dopamine (Tissari, Argiolas, Fadda, Serra, & Gessa, 1979). Norepinephrine concentrations were reduced in the 2-second

shock groups, in agreement with other reports (Dunn et al., in press; Gold & Murphy, 1980).

In hypothalamus re-exposure to fear cues was accompanied by reductions in concentration of both DA and NE. DOPAC was significantly reduced in the 2-second shock groups, but DOPAC:DA ratios did not differ significantly from no shock controls. We found no changes in norepinephrine concentration in amygdala or hippocampus resulting from exposure to stress, despite previous reports of the involvement of noradrenergic mechanisms in these regions related to learning (McGaugh, Liang, Bennett, & Sternberg, 1984; Kovacs et al., 1981).

Of particular interest in the present study were potential differences between diabetic and nondiabetic rats, which might be related to the retention differences found. In this regard, elevations in norepinephrine concentrations were found in diabetics relative to nondiabetics in amygdala, hypothalamus and brainstem. In addition, DOPAC concentration in frontal cortex was significantly lower in diabetics than in nondiabetics, and DOPAC:DA ratios were reduced in diabetic rats in both frontal cortex and hypothalamus. Given that changes in DOPAC concentrations in frontal cortex and hypothalamus and NE and DA concentrations in both frontal cortex and hypothalamus were all sensitive to the effects of the stressors as well, the consistent differences between diabetic and nondiabetic rats in these

measures could be importantly related to the different retention performance.

These neurochemical data strengthen the hypothesis that the distinct physiological state of the diabetic rat, reflected in the present studies in both urinary catecholamine excretion and central catecholamine concentrations, may lead to an enhanced responsivity to the effects of a stressor, such as footshock. The outcome of this is superior retention of a task based upon conditioned fear.

Chapter IV
RESPONSE OF DIABETIC AND NONDIABETIC RATS
TO CHRONIC COLD EXPOSURE

In Experiments 5 and 6, it was demonstrated that diabetic rats had alterations in both urinary catecholamine concentrations and central catecholamine concentrations even prior to being confronted with a learning episode. Moreover, the physiological consequences of the training situation differed in diabetic and nondiabetic rats, as did retention itself. Thus, a single exposure to a stressful stimulus had a different impact upon diabetic rats than it did upon nondiabetic rats, both physiologically and behaviorally. An interaction of stress and metabolic status may similarly be involved in diabetic individuals who experience sudden ketoacidosis subsequent to exposure to a stressor, in spite of taking insulin (MacGillivray et al., 1981). The diabetic rats here were apparently "more susceptible" than nondiabetic rats to responding by avoidance of a stressful stimulus, possibly due, in part, to differences in basal physiologic status and physiologic response to the stressor itself, just as diabetics may be more susceptible or sensitive to the effects of stressors

(Johnson, 1980; Zaidise & Bessman, 1984). In this regard, the animal model was faithful to the clinical situation.

In addition to interacting with metabolic stability in individual episodes, stress may also interact with diabetic control in complex ways during certain extended periods of time. One obvious example of such long-term imposition of additional stress in diabetes is adolescence, a psychologically difficult time, as well as one in which major developmental changes in hormonal status are occurring (Johnson, 1980; Sullivan, 1979). Another time during which stress was noted to have a particularly potent influence upon metabolic stability was during the time of active development of retinopathy (Jacobson et al., 1985). Diabetic individuals experiencing this process reported greater numbers of negative life events, and these events were associated with poorer metabolic control, when compared to diabetics with no retinopathy or to diabetics with established retinopathy. Just as diabetics may have greater difficulty in coping with individual stressful situations, they may also suffer greater negative consequences when confronting stresses of longer duration.

Experiment 7 was conducted to evaluate the effects of a longer lasting stressor in the streptozotocin diabetic rat. The same hormonal and neurochemical responses were evaluated here that were evaluated following footshock stress.

Experiment 7. Plasma Corticosterone Concentrations,
Catecholamine Excretion and Central Catecholamine
Concentrations in Diabetic and Nondiabetic Rats
Exposed to Chronic Cold

Method

Animals and housing

A total of 30 adult male Sprague-Dawley rats were involved in this experiment. Half the rats were made diabetic with a single IP injection of streptozotocin as described before. The remaining rats were injected with vehicle, and the experiment was conducted one month after diabetes induction. The rats were housed and maintained as previously described except for the 72 hour period of cold exposure. During this time they were housed singly in standard cages in a ventilated cold room which was maintained at 4°C and which had an artificial light/dark cycle identical to that of the vivarium.

Procedure

Two experimental periods of three days each were conducted. During each, 6 diabetic (DI) and 6 nondiabetic rats (NI) were given 4U of PZI twice daily. Two other groups (DS and NS) received saline injections. The high insulin dose led to the attrition of a few nondiabetic rats, and some diabetic rats were also lost in the cold. Enough rats were used initially to have final Ns of 6 in each condition. During the first (warm) measurement period, the rats remained in the vivarium. During the second period, they were in the cold room. During the third day of insulin

administration in the warm measurement period, the rats were placed on a metabolism rack in their home cages for collection of urine. The rats were housed on this same metabolism rack the entire time they were in the cold room, and urine was collected in the cold during both the first and the last 24 hour periods.

At the end of the first measurement period, 1 ml of blood was collected from the rats under light ether anesthesia by cardiac puncture. For one week following the insulin administration, urine collection and blood collection in the warm, the rats were left untreated. They were then moved to the cold room and insulin administration was again given to half the rats. At the end of this second measurement period (in the cold), the rats were killed by decapitation, blood was collected from the cervical wound and brains were removed and regions dissected. Blood collection and decapitation were carried out between 9:00 and 10:00 A.M.

Plasma corticosterone determinations

Blood samples taken by cardiac puncture in the first measurement period and from the cervical wound in the second measurement period were placed in heparinized tubes. The blood was centrifuged for 10 minutes, and the plasma was removed and stored at -70°C until assayed for corticosterone. The protein-binding assay (Murphy, 1967) was described in detail in Experiment 4. Mean

concentrations in the 4 groups were compared using two-way ANOVA.

Urine catecholamine measurement

Urine was collected in beakers containing 0.5 ml of 3N HCL. At the end of each of the 3 measurement periods, urine volume was measured and urine was frozen until assayed for catecholamines. The same procedures and equipment were utilized for these measurements as were used in experiment 5. Urine creatinine was also measured for each collection period in this experiment, and plasma creatinine concentration was also measured in the plasma collected at the end of the cold measurement period, so that GFR at the end of 3 days cold exposure could be calculated. Because the same rats were not included in all three measurements due to attrition, separate two-way ANOVAs were used to compare the 4 groups in the warm, acute cold and chronic cold conditions.

Neurochemical determinations

At the end of the cold measurement period, following sacrifice of the rats by cervical dislocation, brains were quickly removed and placed on an ice cold petri dish and the following brain regions were dissected and frozen until later biochemical assays: frontal cortex (69.6 ± 3.3 mg), hypothalamus (58.1 ± 1.9 mg), amygdala (84.5 ± 3.6 mg), hippocampus (111.9 ± 4.9 mg) and brainstem (176.2 ± 5.4 mg). The disparity in tissue weights between this experiment and

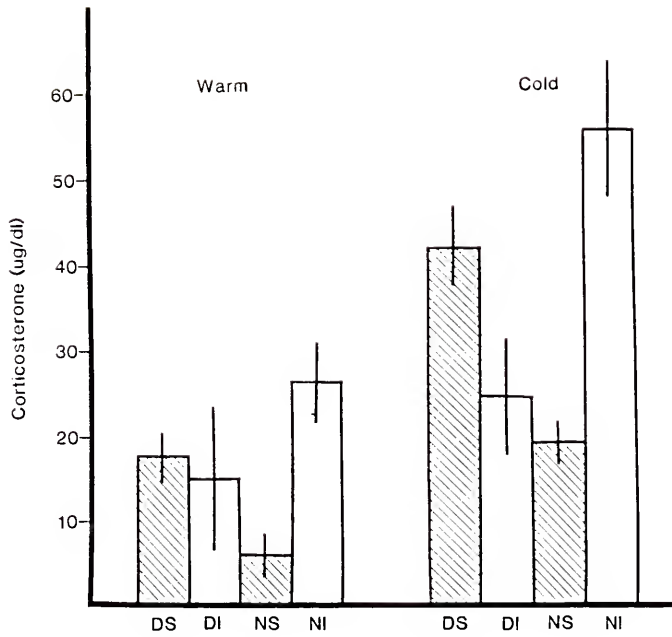
Experiment 6 was due to preparation of tissues by different individuals in the two studies. The tissues were analyzed by HPLC for concentrations of NE, DA and DOPAC. All the details of the various procedures involved in the neurochemical analyses were discussed in Experiment 6. Effects of condition and of insulin treatment upon regional catecholamine concentrations were compared with two-way ANOVA.

Results and Discussion

Plasma corticosterone determinations

Mean plasma corticosterone concentrations are shown in Figure 19. Treatment (saline vs insulin) exerted significant effects ($F(1,32)=7.03$, $P<0.02$), as did temperature (cold vs warm, $F(1,32)=30.12$, $P<0.005$). There were interactive effects of condition (diabetic vs nondiabetic) and treatment ($F(1,32)=30.70$, $P<0.005$). A significant interaction ($F(1,32)=4.77$, $P<0.04$) among all three factors--condition, insulin treatment, and cold--was also found. Summarizing these rather complex results, saline-treated nondiabetic rats in the warm environment (baseline) had corticosterone concentrations quite similar to the previously reported baseline values in Experiment 4. Both diabetic groups had slightly higher corticosterone concentrations in the warm environment than did the nondiabetic saline group, possibly due to handling 1 hour prior to blood collection during morning insulin

Figure 19. Mean plasma corticosterone concentrations of saline-treated and insulin-treated diabetic and nondiabetic rats at 23°C and after 72 hours at 4°C. DS=diabetic saline-treated, DI=diabetic insulin-treated, NS=nondiabetic saline-treated, NI=nondiabetic insulin-treated. There was a significant 3 way interactive effect of condition (diabetics vs nondiabetics), treatment (insulin vs saline) and temperature (cold vs warm).



administration. The insulin-treated nondiabetic group had a still higher mean corticosterone concentration, which may reflect the metabolic response to hypoglycemia.

After 3 days in the cold, all the groups had elevated corticosterone concentrations, relative to their respective warm baseline values. Insulin-treated diabetics and saline-treated nondiabetics had smaller increments than did the other groups. Saline-treated diabetics had corticosterone concentrations similar to those found in response to re-exposure to fear cues in experiment 4. That is, after 3 days of exposure to cold stress there did not appear to be any adaptation of plasma corticosterone concentration in these rats. Insulin-treated diabetics and saline-treated nondiabetics, on the other hand, may have had lower corticosterone concentrations due to adaptation to cold, although this possibility was not directly tested in the present experiment. Insulin-treated nondiabetic rats had very high corticosterone concentrations after 3 days in the cold, possibly due to additive effects of insulin-induced hypoglycemia and cold exposure.

The corticosterone concentrations of saline-treated diabetics in the warm seemed high. These morning concentrations were much higher than the concentrations found in undisturbed diabetic and nondiabetic rats in the afternoon. There are reports of higher basal corticosterone concentrations in diabetic rats than in

nondiabetics (L'Age et al., 1974; Tornello et al., 1981b), but these differences occurred in the afternoon, at least in one case (Tornello et al., 1981b). In the present investigation, morning insulin and saline injections, given 2 hours prior to blood sampling, may have produced increments in corticosterone in the diabetic rats which were not totally restored to normal by the time of blood collection. The extremely high corticosterone concentrations in the insulin-treated nondiabetics suggest that the hypoglycemia produced by insulin acted as a stressor (Gann, Dallman, & Engeland, 1981).

The chronic cold data suggest that, in general, corticosterone concentrations tend to remain elevated above baseline for extended periods in the face of a continuing stressor. The saline-treated diabetics had higher concentrations than did insulin-treated diabetics and saline-treated nondiabetics. While in the present experiment there were no measurements of corticosterone increments with acute exposure to cold, a previous report (Odio & Maickel, 1985) found corticosterone concentrations of about 45 ug/dl in untreated rats 1 hour after exposure to cold, with reductions to about 30 ug/dl after 4 hours. Thus, in the present experiment, insulin-treated diabetics and saline-treated nondiabetics may have adapted somewhat to the cold stressor after 3 days, whereas the saline-treated diabetics did not. Insulin-treated nondiabetic rats had

very high corticosterone concentrations, again likely indicating an additive effect of two stressors-- hypoglycemia and cold. It is interesting in this regard that among the nondiabetic state dependent learning groups, rats which were receiving insulin at the time of re-exposure to fear cues (SI) also had extremely high corticosterone concentrations. Rats receiving insulin at the time of footshock training (IS) did not have such extreme elevations in corticosterone. It would thus seem that there are additive effects of insulin-induced hypoglycemia and psychological stress (conditioned fear) and of hypoglycemia and cold exposure, but not of hypoglycemia and footshock.

Urine catecholamine measurement

In Table 5, baseline values from Experiments 5 and 7 are compared. Table 6 shows body weights, total urine creatinine excretion, plasma creatinine concentrations and GFRs of the 4 groups. A significant interactive effect of condition and insulin treatment was noted for urine creatinine excretion in the warm and chronic cold (warm-- $F(1,20)=9.82$, $P<0.01$; chronic cold-- $F(1,19)=6.71$, $P<0.02$). Diabetics had higher plasma creatinine concentrations than did nondiabetics ($F(1,19)=59.05$, $P<0.001$), and insulin treatment also led to elevations in plasma creatinine concentration ($F(1,19)=15.31$, $P<0.01$). Condition and insulin treatment had an interactive effect on GFR ($F(1,19)=5.33$, $P<0.04$). Thus, diabetics had reduced GFR

TABLE 5

Comparison of Two Measurements of Baseline Twenty-Four Hour Catecholamine Excretion in Diabetic and Nondiabetic Rats.

	WARM		BASELINE	
	Diab	NDiab	Diab	NDiab
DOPAMINE				
Creatinine ^a	236±45	257±39	223±23	270±24
Body weight ^b	13.5±2.6	8.6±1.6	15.8±1.7	9.1±.69
Total ^c	5.0±.96	4.3±.69	6.4±.79	4.7±.32
NOREPINEPHRINE				
Creatinine	82±11	66±9	77±11	57±5
Body weight	4.7±.66	2.2±.37	5.5±.87	1.9±.15
Total	1.7±.23	1.1±.17	2.3±.40	1.0±.08
EPINEPHRINE				
Creatinine	--	13±2	9±1	21±3
Body weight	--	.41±.07	.62±.05	.69±.08
Total	--	.21±.03	.25±.02	.36±.04

^ang/mg creatinine

^bug/kg body weight

^cug/24 hours

TABLE 6
Renal Variables in Cold Exposed Rats

Group	Variable	Days of Cold Exposure		
		0	1	3
<u>SALINE</u>				
	<u>Nondiabetics</u>			
	Bwt	513±23	-	-
	P _{Cr}	-	-	0.8±0.04
	UV	24.3±3.3	38.3±4.4	92.2±16.2
	UV _{Cr}	16.9±1.1	20.8±1.3	22.3±1.5
	GFR	-	-	2.03±0.16
	<u>Diabetics</u>			
	Bwt	385±22	-	-
	P _{Cr}	-	-	1.6±0.14
	UV	184±10	218±15	269±28
	UV _{Cr}	21.5±0.9	21.7±0.6	21.9±1.0
	GFR	-	-	1.00±0.11
<u>INSULIN</u>				
	<u>Nondiabetics</u>			
	Bwt	523±29	-	-
	P _{Cr}	-	-	0.6±0.04
	UV	39.5±6.8	36.0±3.8	85.0±6.3
	UV _{Cr}	20.2±2.3	20.8±2.9	29.9±2.1
	GFR	-	-	3.52±0.43
	<u>Diabetics</u>			
	Bwt	322±17	-	-
	P _{Cr}	-	-	1.1±0.12
	UV	61.8±19.6	156±22	108±12
	UV _{Cr}	16.5±1.0	23.0±1.6	21.4±1.8
	GFR	-	-	1.42±0.14

Bwt, body weight (g)

P_{Cr}, plasma creatinine concentration (mg/dl)

UV, 24 hour urinary volume (ml)

UV_{Cr}, 24 hour urinary creatinine excretion (mg)

GFR, glomerular filtration rate (ml/min)

relative to nondiabetics, with both diabetic and nondiabetic insulin-treated groups having higher GFR than their saline-treated counterparts.

Urine catecholamine data were again expressed as excretion normalized to body weight, as excretion normalized to creatinine excretion, and as total excretion. Figures 20, 21 and 22 show urine norepinephrine excretion. There were significant interactive effects of condition (diabetic vs nondiabetic) and treatment (insulin vs saline) upon norepinephrine excretion calculated with the body weight normalizations or as total excretion (body weight-- $F(1,20)=7.82$, $P<0.02$; total excretion-- $F(1,20)=9.12$, $P<0.01$). Calculations with the normalization to creatinine just failed to reach significance at the 0.05 level of significance ($F(1,20)=3.76$, $P=0.064$). Thus, saline-treated diabetics had greater norepinephrine excretion (in agreement with the passive avoidance data), while insulin had opposite effects on diabetic and nondiabetic groups. In the acute cold, there were significant effects of both condition and treatment on norepinephrine excretion with all three measures, and with the creatinine normalization and total excretion there were also interactive effects of condition and treatment (body weight, condition-- $F(1,19)=40.66$, $P<0.001$; treatment-- $F(1,19)=6.88$, $P<0.02$; creatinine-- $F(1,19)=13.70$, $P<0.01$; total excretion-- $F(1,19)=6.53$, $P<0.02$). Saline-treated diabetic rats had particularly

Figure 20. Urinary excretion of norepinephrine (ug excreted per Kg body weight) during 24 hours at 23°C, during the first 24 hours exposed to 4°C, and during the last 24 of 72 hours exposed to 4°C. DI=diabetic insulin-treated, DS=diabetic saline-treated, NI=nondiabetic insulin-treated, NS=nondiabetic saline-treated. +Warm--interactive effect of condition (diabetics vs nondiabetics) and treatment (insulin vs saline). *Acute cold--significant main effects of condition and treatment.

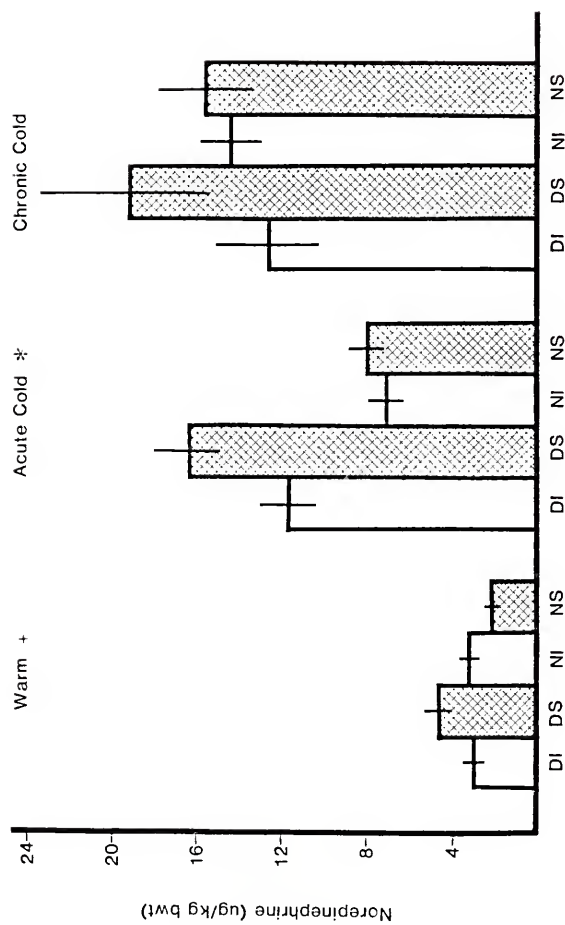


Figure 21. Urinary excretion of norepinephrine (ng/mg creatinine) during 24 hours at 23°C, during the first 24 hours exposed to 4°C, and during the last 24 of 72 hours exposed to 4°C. DI=diabetic insulin-treated, DS=diabetic saline-treated, NI=nondiabetic insulin-treated, NS=nondiabetic saline-treated. *Acute cold--significant main effects of condition (diabetics vs nondiabetics) and treatment (insulin vs saline). +Acute cold--interactive effect of condition and treatment. *Chronic cold--significant effect of treatment.

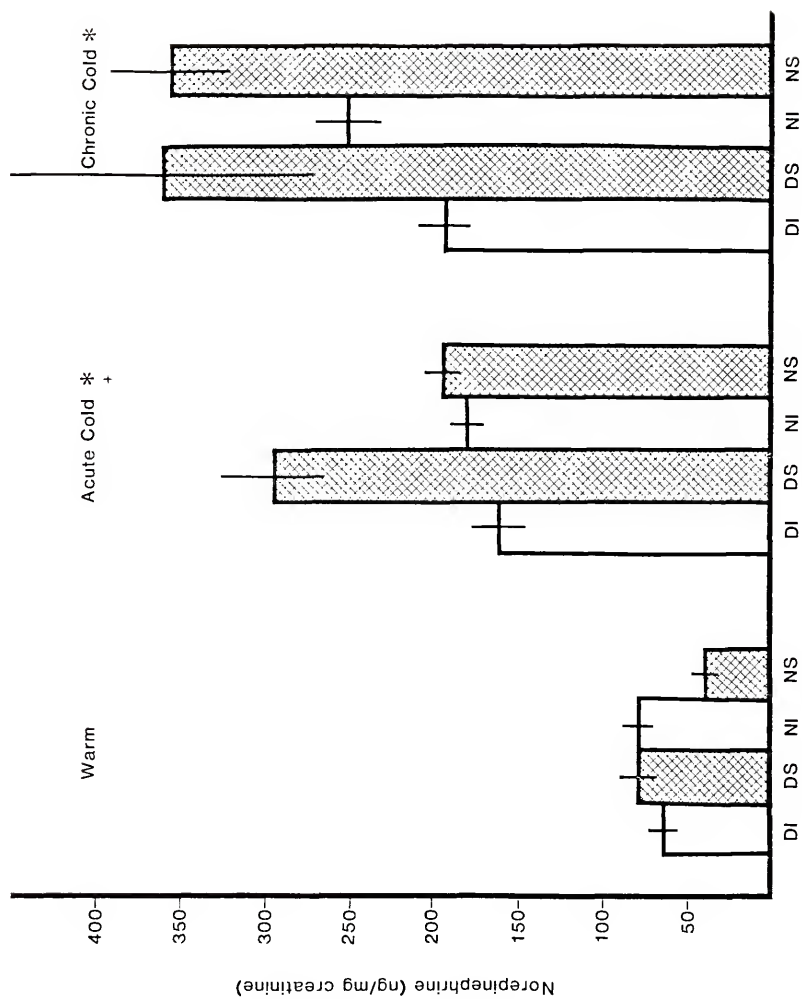
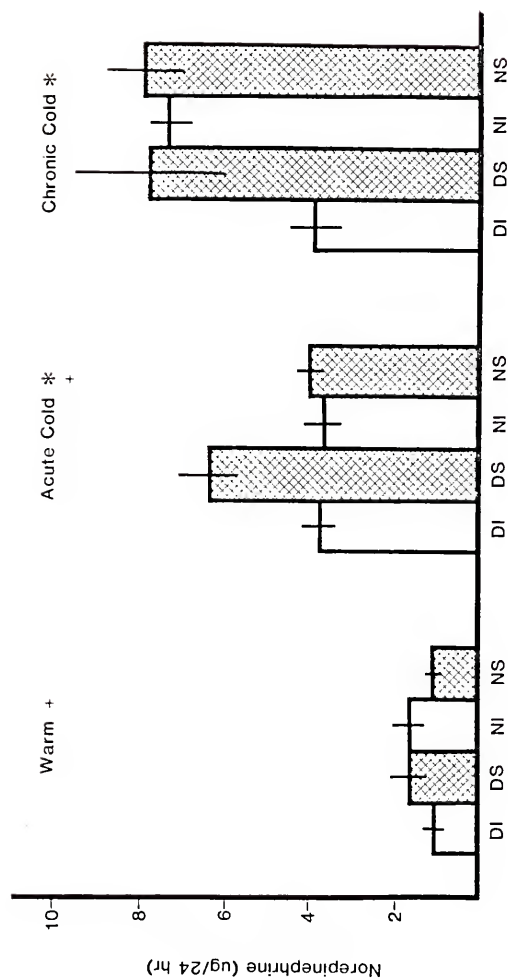


Figure 22. Urinary excretion of norepinephrine (total ug excreted per 24 hours) during 24 hours at 23°C, during the first 24 hours exposed to 4°C, and during the last 24 of 72 hours exposed to 4°C. DI=diabetic insulin-treated, DS=diabetic saline-treated, NI=nondiabetic insulin-treated, NS=nondiabetic saline-treated. +Warm--interactive effect of condition (diabetics vs nondiabetics) and treatment (insulin vs saline). *Acute cold--significant main effects of condition and treatment +Acute cold--interactive effect of condition and treatment. *Chronic cold--significant effect of treatment.



vigorous responses to acute cold stress. In the chronic cold, the body weight normalization revealed no significant effects, while normalization to creatinine and total excretion indicated significant effects of insulin treatment on norepinephrine excretion (creatinine-- $F(1,19)=11.31$, $P<0.01$; total excretion-- $F(1,19)=5.99$, $P<0.03$). Thus, at this time insulin was suppressing the usual continuing rise in norepinephrine excretion during extended cold exposure (Roy et al., 1977).

Figures 23, 24 and 25 show dopamine excretion. There were no significant differences among the groups in the warm by any measure of dopamine excretion. In the acute cold exposure, the body weight normalization showed a significant effect of condition ($F(1,19)=24.29$, $P<0.001$), while with the creatinine normalization there was an interactive effect of condition and insulin treatment ($F(1,19)=4.27$, $P=0.05$). There were no significant effects in the total excretion measurement. In the chronic cold, there were significant differences in dopamine excretion in diabetics and nondiabetics measured by the creatinine normalization or by total excretion (creatinine-- $F(1,19)=10.54$, $P<0.01$; total excretion-- $F(1,19)=14.45$, $P<0.01$). Clearly, no significant differences in dopamine excretion were consistently found in conjunction with chronic exposure to a cold environment.

Problems in ascertaining epinephrine values in the diabetic groups prevented analysis of this catecholamine.

Figure 23. Urinary excretion of dopamine (ug excreted per Kg body weight) during 24 hours at 23°C, during the first 24 hours exposed to 4°C, and during the last 24 of 72 hours exposed to 4°C. DI=diabetic insulin-treated, DS=diabetic saline-treated, NI=nondiabetic insulin-treated, NS=nondiabetic saline-treated. *Acute cold--significant effect of condition (diabetics vs nondiabetics).

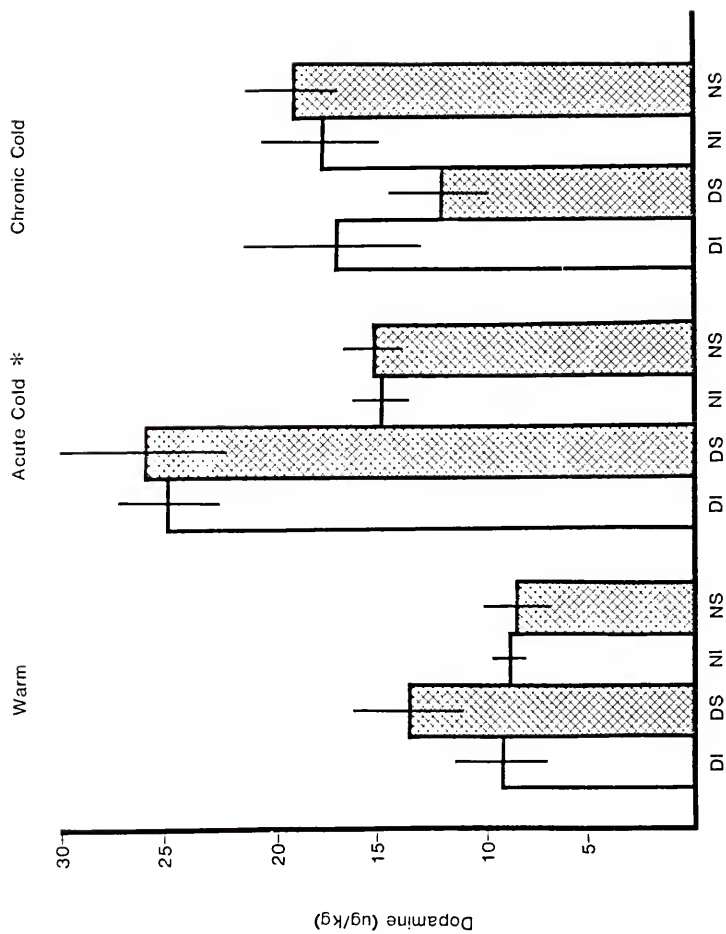


Figure 24. Urinary excretion of dopamine (ng/mg creatinine) during 24 hours at 23°C, during the first 24 hours exposed to 4°C, and during the last 24 of 72 hours exposed to 4°C. DI=diabetic insulin-treated, DS=diabetic saline-treated, NI=nondiabetic insulin-treated, NS=nondiabetic saline-treated. +Acute cold--interactive effect of condition (diabetics vs nondiabetics) and treatment (insulin vs saline). *Chronic cold--significant effect of condition.

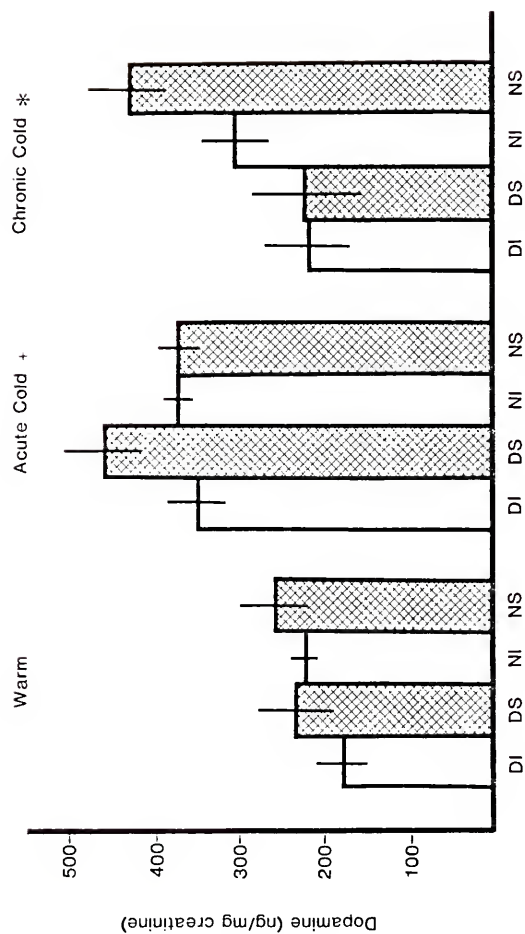
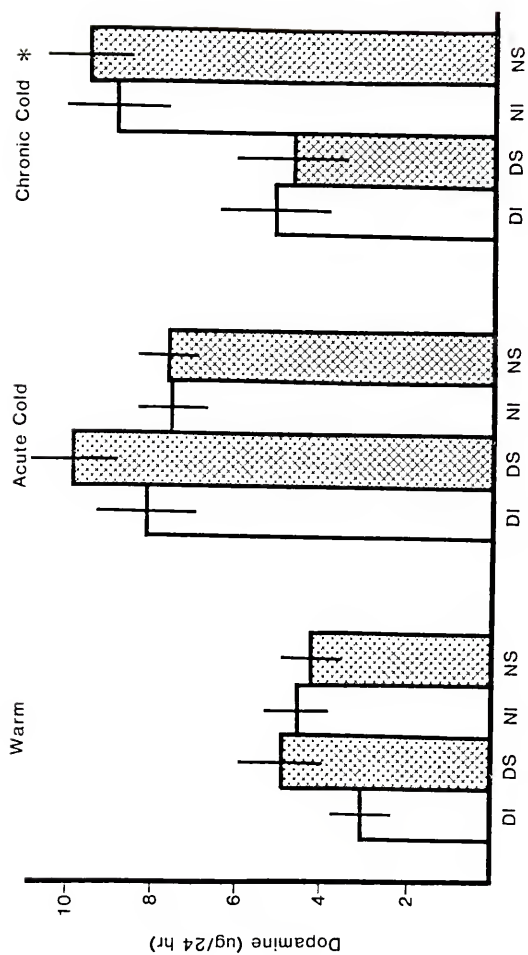


Figure 25. Urinary excretion of dopamine (total ug excreted per 24 hours) during 24 hours at 23°C, during the first 24 hours exposed to 4°C, and during the last 24 of 72 hours exposed to 4°C. DI=diabetic insulin-treated, DS=diabetic saline-treated, NI=nondiabetic insulin-treated, NS=nondiabetic saline-treated. *Chronic cold--significant effect of condition (diabetics vs nondiabetics).



Nonetheless, there were some important differences found in norepinephrine excretion. First, in the warm, saline-treated diabetics had greater excretion than did saline-treated nondiabetics. This finding replicated the finding of increased norepinephrine excretion in diabetic rats in Experiment 6. The elevation in norepinephrine excretion in the insulin-treated nondiabetic rats in the warm was likely the result of the stress of hypoglycemia. As would be expected, the cold exposure led to large increases in norepinephrine, certainly as a result of increased sympathetic activity in response to this stress. The response was exaggerated in the saline-treated diabetic rats. Cold combined with insulin treatment did not seem to produce an additive effect upon norepinephrine. If anything, insulin attenuated the response of norepinephrine to chronic cold exposure.

The most dramatic finding in dopamine excretion was the reduction in saline-treated diabetics after 3 days in the cold, although the reliability of the dopamine data is weakened by the problems of normalization. However, the results based upon the creatinine normalization and total excretion suggest that the diabetic rats were unable to sustain the same level of excretion of dopamine after chronic stress.

Because there were differences in apparent GFR among the groups in this experiment, the meaning of differences in

catecholamine excretion must be interpreted with caution. Additional investigation of plasma and peripheral tissue concentrations of the catecholamine would be helpful in clarifying these differences.

Neurochemical determinations

Figure 26 shows NE concentrations in amygdala and hippocampus. For comparison, concentrations of the no shock controls from experiment 6 were included in the figures. However, these groups were not included in the statistical analyses and were only included as reference points. There were no significant differences in either norepinephrine concentrations in either amygdala or hippocampus among the cold stress groups.

There were a number of significant differences in the frontal cortex (Figure 27). Condition (diabetic vs nondiabetic) and treatment (insulin vs saline) had an interactive effect on DA (upper panel, $F(1,20)=6.41$, $P<0.03$), whereby insulin-treated diabetics had higher DA concentrations than their saline-treated counterparts, while insulin-treated nondiabetics had lower DA concentrations than saline-treated nondiabetics. DOPAC to DA ratios were increased in insulin-treated groups ($F(1,12)=5.9$, $P<0.04$). Concentration of NE (lower panel) was significantly higher in diabetics ($F(1,23)=11.35$, $P<0.01$). Insulin treatment produced reduced NE concentrations in both diabetic and nondiabetic groups, although this effect just missed the

Figure 26. Upper panel: Norepinephrine concentrations in amygdala of nondiabetic and diabetic rats sacrificed after 72 hours exposed to 4°C. sal=saline-treated, ins=insulin-treated, bas=no exposure controls from learning experiment, for comparison.

Lower panel: Norepinephrine concentrations in hippocampus. No significant differences.

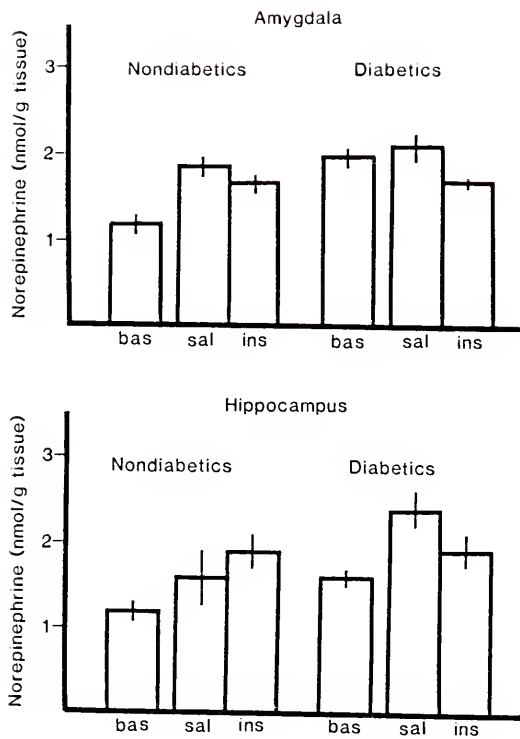
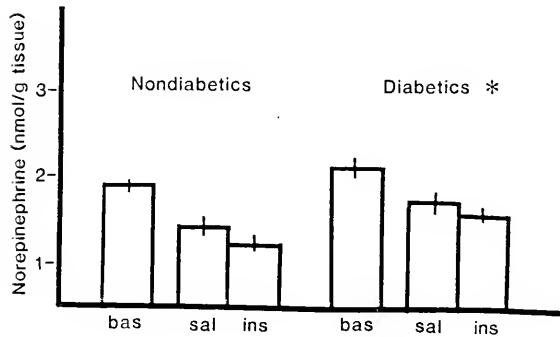
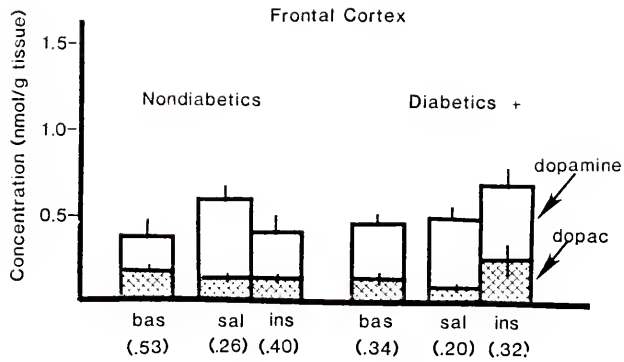


Figure 27. Upper panel: Dopamine (clear bars) and DOPAC (textured bars) concentrations in frontal cortex of rats sacrificed after 72 hours at 4°C, DOPAC to dopamine ratios in parentheses. sal=saline-treated, ins=insulin-treated, bas=no exposure controls from learning experiment, for comparison. +Interactive effect of condition (diabetics vs nondiabetics) and treatment (insulin vs saline) on dopamine concentration.

Lower panel: Norepinephrine concentrations. *Diabetics significantly different from nondiabetics.



usual accepted level of significance ($F(1,23)=4.18$, $P=0.054$). Although, it is not possible to make direct comparisons of the NE values in the cold experiment with the baseline values from the passive avoidance experiment, it is interesting to note that concentrations of the amine do appear to be reduced following chronic cold exposure.

In the hypothalamus (Figure 28, upper panel), condition and insulin treatment had independent effects upon the DOPAC to DA ratio. Diabetics had lower ratios than did nondiabetics ($F(1,23)=4.49$, $P<0.05$), and insulin-treated groups (both diabetic and nondiabetic) had higher ratios than did saline-treated groups ($F(1,23)=11.15$, $P<0.01$). There were no significant differences in NE (lower panel) between diabetics and nondiabetics. Moreover, qualitative comparison with baseline values suggests no differences in concentrations of hypothalamic NE following 3 days of chronic cold.

In the brainstem (Figure 29, upper panel), diabetics had significantly lower DOPAC to DA ratios here as in the hypothalamus ($F(1,18)=7.18$, $P<0.02$), and there was an interactive effect of condition and insulin treatment on this ratio ($F(1,18)=7.67$, $P<0.02$), with insulin leading to increases in the ratio to a much greater extent in nondiabetics than in diabetics. Diabetics had higher NE concentrations (lower panel) than nondiabetics ($F(1,23)=6.76$, $P<0.02$).

Figure 28. Upper panel: Dopamine (clear bars) and DOPAC (textured bars) concentrations in hypothalamus of rats sacrificed after 72 hours at 4°C. sal=saline-treated, ins=insulin-treated, bas=no exposure controls from learning experiment, for comparison. Interactive effects of condition (diabetics vs nondiabetics) and treatment (insulin vs saline) on DOPAC to DA ratio.

Lower panel: Norepinephrine concentrations.

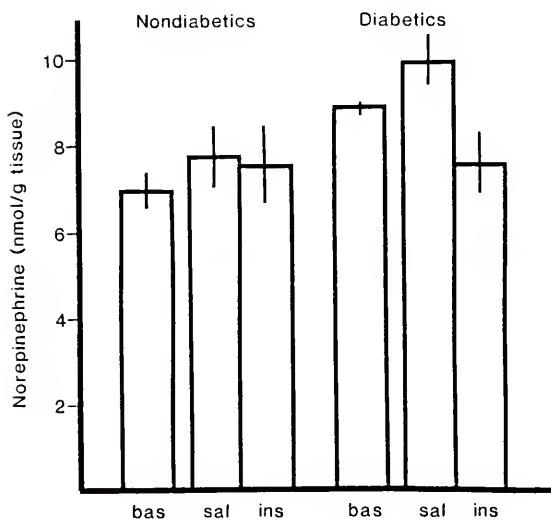
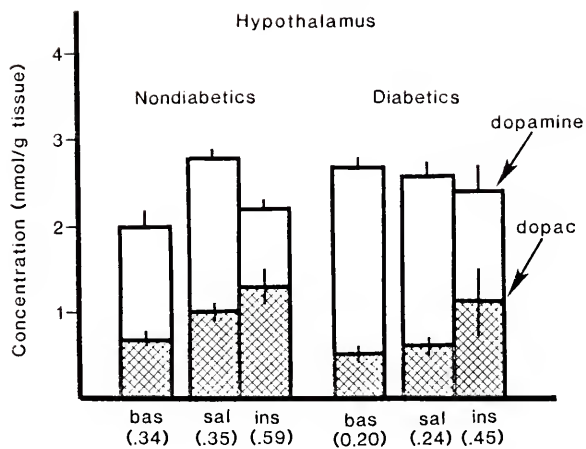
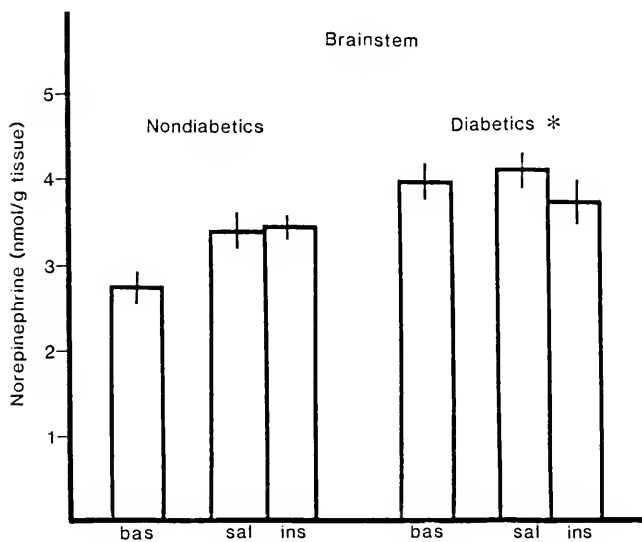
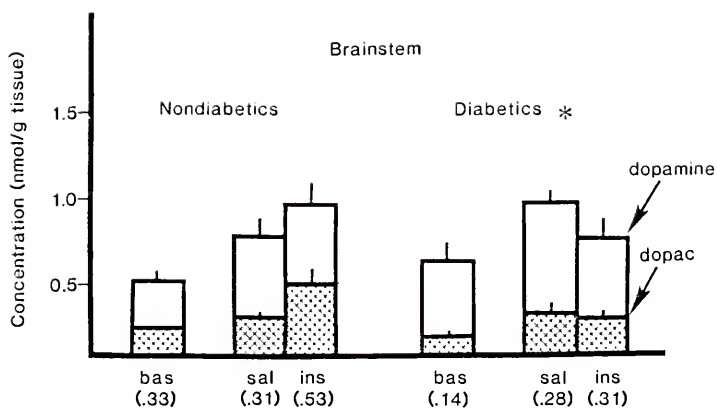


Figure 29. Upper panel: Dopamine (clear bars) and DOPAC (textured bars) concentrations in brainstem of rats sacrificed after 72 hours at 4°C. sal=saline-treated, ins=insulin-treated, bas=no exposure controls from learning experiment, for comparison. *Significant main effect of condition (diabetics vs nondiabetics) and significant interactive effect of condition and treatment (insulin vs saline) on DOPAC concentration.

Lower panel: Norepinephrine concentrations. *Diabetics significantly different from nondiabetics.



Reviewing the major effects of chronic cold stress on regional catecholamine concentrations, there were effects of both diabetes and of insulin in several regions. In frontal cortex, DA concentrations were affected in opposite ways by insulin in diabetics and nondiabetics, that is, DA concentrations were higher in insulin-treated diabetics and lower in insulin-treated nondiabetics than in their respective saline-treated counterparts. Insulin increased DOPAC:DA ratios (and presumably therefore dopamine turnover) in both diabetic and nondiabetic rats. Norepinephrine concentrations in frontal cortex were significantly higher in diabetic rats, and the reduction of NE concentration by insulin approached significance.

In hypothalamus, diabetics had lower DOPAC:DA ratios than did nondiabetics, and this was also found in the passive avoidance experiment. As in frontal cortex, insulin increased DOPAC:DA ratios in hypothalamus in both diabetic and nondiabetic rats.

In brainstem, there was the same enhancement of DOPAC:DA ratios by insulin, whereby diabetics had lower ratios than nondiabetics in this region as they did in hypothalamus. Concentration of NE in brainstem was higher in diabetics than in nondiabetics, and again, the same was true in passive avoidance groups.

With chronic cold stress, diabetics continued to show elevations in NE concentrations in some regions, and reduced

DA turnover, relative to nondiabetics. The additional finding in the present experiment is the effect of insulin upon dopamine turnover, and possibly upon NE concentration in frontal cortex. The data demonstrate that exogenous insulin administration in diabetics does not totally normalize neurochemical response to cold stress. Rather, insulin may have effects upon catecholamine concentration independent of its effects upon plasma glucose concentration. Unfortunately, the present experiment did not include its own baseline groups. It would have been helpful to have included both diabetic and nondiabetic groups receiving both saline and insulin but receiving no exposure to cold to determine if insulin would normalize basal catecholamine concentrations in the absence of any stressors.

While no behavioral correlates of the hormonal and neurochemical adaptations to cold were examined in the present study, the results indicate that such behavioral assays are warranted in future studies. The present results suggest that diabetes mellitus is accompanied by numerous alterations in both peripheral and central catecholamines and that these are not normalized by insulin replacement, at least during periods when exposure to chronic stress is encountered.

CHAPTER V GENERAL DISCUSSION

The major behavioral finding of these experiments is the significantly better performance of diabetic rats compared with nondiabetic rats in retention of passive avoidance. Preliminary experiments ruled out the possibility that diabetic rats would simply be less active or that they would respond differently than nondiabetic rats to footshock. Thus, the difference in passive avoidance responding would seem to represent actual associative differences between diabetic and nondiabetic rats.

The superiority of the diabetic rats was somewhat unexpected, given the clinical finding of impaired performance of diabetic adolescents in a number of neuropsychological tasks (Ryan et al., 1985). However, the present task as evaluated in rats, was certainly not directly analogous to human learning situations. Rather, it represented a situation where fear provided the motivation for learning an avoidance response. Although having a somewhat limited correspondence with human learning, passive avoidance is a task often used to investigate memory in animal models, and it has proven useful in establishing hormonal and neurochemical correlates of memory storage.

Evaluated in this light, the present results suggest a number of differences in the diabetic rats which may have predisposed them to better retention of the stressful task. Increases in plasma epinephrine have been shown to occur following footshock strong enough to lead to later avoidance (2 mA) but not following weak (0.6 mA) footshock (McCarty & Gold, 1981). Norepinephrine concentrations in plasma also increase following footshock.

While not pinpointing specific temporal changes in epinephrine and norepinephrine in response to footshock, Experiment 5 demonstrated increased excretion of epinephrine in diabetic rats during the 24 hours following footshock training, while excretion in the nondiabetic rats was reduced during this same interval. Moreover, norepinephrine excretion was elevated in diabetic rats irrespective of footshock training.

In addition to these peripheral hormonal differences between diabetic and nondiabetic rats, a number of neurochemical differences also were found. For example, dopamine concentration in frontal cortex following footshock training was significantly higher in diabetic than in nondiabetic rats. Comparing the footshock retention groups, diabetic rats had higher norepinephrine concentrations than nondiabetic rats in amygdala, hypothalamus and brainstem, and higher dopamine concentrations in hypothalamus, independent of re-exposure to fear cues. Diabetics also had lower DOPAC

concentrations in frontal cortex and lower DOPAC:DA ratios in frontal cortex and hypothalamus. Finally, Only diabetic rats exposed to fear cues had reductions in brainstem norepinephrine concentrations, a finding previously associated with stress (Dunn et al., in press). It is possible that these differences reflect adaptive changes in central monoamine systems to hyperglycemia (Bitar et al., 1986).

These neurochemical distinctions in the diabetic rats, in addition to the peripheral catecholamine differences, provided a vastly different biochemical state in the diabetic rats as they approached the training situation, and this difference in baseline state would be expected to contribute to the subsequent responses, both behavioral and hormonal (Leshner, 1975). The biochemical state of diabetics was particularly relevant to the aversive training situation, given that the hormones modulated were those known to be involved in the mechanisms of memory storage (Gold & McGaugh, 1978).

It has been suggested that the metabolic state produced in uncontrolled diabetes is similar to that of chronic stress (Zaidise & Bessman, 1984). Furthermore, exposure to stress can lead to sensitization of amine systems (Glavin, 1985). Thus, the diabetic may be predisposed to respond to stress; that is, if stress hormones are already elevated in the diabetic upon entering a stressful situation, there may

be additive effects of initial state and stress-induced increments of some hormones. Such effects in clinical situations may be responsible for the diabetic perceiving events negative life events as more stressful (Linn et al., 1983). Sensitization of the stress response is also a contributing factor in metabolic decompensation (Shamoon et al., 1980; MacGillivray et al., 1981).

In the present experiments, sensitization of stress responses would also help to account for the superior performance of diabetics in passive avoidance retention in the present studies. A state similar to chronic stress, as reflected in peripheral catecholamine alterations (Berkowitz et al, 1980 and the present results), may exist in diabetic rats. They might, then, respond with greater increments in stress hormones than nondiabetics to a given amount of footshock. Since the amount of footshock is a determinant of retention (Ader et al., 1972), as well as of hormonal response (Friedman et al., 1967), diabetics would then be expected to exhibit better retention than nondiabetics.

Clearly, some of the neurochemical changes were not confined to the diabetic groups, but rather were correlates of the conditioned fear accompanying re-exposure to the training apparatus. In frontal cortex, concentrations of both DA and DOPAC were elevated in re-exposed groups, although DOPAC:DA ratios were not elevated in the present study. The elevation in DOPAC concentration is consistent

with other reports of conditioned fear (Herman et al., 1982; Dunn et al., in press).

Hypothalamic DA and NE concentrations were reduced in rats returned to the training apparatus. Reductions in NE concentration in hypothalamus have previously been reported to occur following re-exposure to both a footshock training apparatus and to footshock itself (Dunn et al., in press; Anisman & Sklar, 1979). Thus, the present neurochemical results are generally consistent with previous reports concerning monoamine responses to stress, but in addition demonstrate a number of differences in diabetic rats in this regard.

The results of the cold stress manipulation confirm that the differences in response to stress seen in diabetic rats is not restricted to footshock. Larger increments in urinary norepinephrine excretion were observed in diabetics during the first 24 hours of exposure to cold, with particularly vigorous responses in saline-treated diabetic rats.

After three days in the cold, differences were found between diabetic and nondiabetic rats in concentration of NE in frontal cortex and brainstem, with diabetics having higher concentrations in both tissues. Diabetics also had lower DOPAC:DA ratios in hypothalamus than did nondiabetics. Unfortunately, the cold stress experiment did not include neurochemical determinations of animals not exposed to cold

and so no conclusions can be made as to the relative ability of diabetics and nondiabetics to adapt to 3 days in a cold environment. However, an important finding of this experiment was that insulin had both independent and interactive effects upon metabolic parameters. These included opposite effects upon norepinephrine excretion in diabetic and nondiabetic rats in the warm environment, reduction of urinary concentrations in diabetics and increased concentrations in nondiabetics. After 3 days in the cold, insulin-treated groups had lower urinary NE excretion than did saline-treated groups.

In brain, insulin had opposite effects upon concentration of DA in frontal cortex of diabetic and nondiabetic rats, producing higher concentrations in diabetics but lower concentrations in nondiabetics. Insulin treatment in association with cold exposure also produced higher DOPAC:DA ratios in frontal cortex, hypothalamus and brainstem. Thus, insulin replacement in diabetics cannot be assumed merely to normalize the metabolic state of these individuals. Exogenous insulin administration at widely spaced intervals does not duplicate the normal endogenous release of insulin and may, indeed, produce a whole range of alterations in hormonal concentrations.

The present results demonstrated better retention for passive avoidance in diabetic rats and a number of hormonal and neurochemical differences which may help to account for

the behavioral differences. It would be of interest in future experiments to extend the behavioral findings to additional learning situations. In particular, it would be instructive to determine if appetitive learning, which presumably is not as stressful as passive avoidance, would be different in diabetic and nondiabetic rats.

It would also be interesting to determine if extended exposure to stress, as in the 72 hours of cold exposure here, has an influence on behavioral variables, since the present results indicated more rapid response of norepinephrine excretion to cold in diabetic rats and an apparent depletion in renal dopamine production after 72 hours. The cold environment also led to significantly higher concentrations of norepinephrine in frontal cortex and brainstem of diabetics. In addition, diabetics had reduced DOPAC to DA ratios in frontal cortex and brainstem and reduced DOPAC concentration in brainstem. These neurochemical differences, added to the differences noted in peripheral catecholamine activity associated with chronic stress may well have behavioral consequences. Since there are certain times when long-term stress and metabolic parameters interact potently in diabetics (Johnson, 1980; Sullivan, 1979; Jacobson et al., 1985), such behavioral studies would be of clinical relevance.

In addition to providing data relevant to clinical issues, the streptozotocin-diabetic rat provides an

interesting model for comparison with other rats in the study of hormonal and neurochemical correlates of memory storage. The present results indicated that better retention in diabetics was associated with hormonal and neurochemical alterations previously shown to correlate with memory formation (McCarty & Gold, 1981; Herman et al., 1982). More recently, glycemic state itself has been implicated in the processing of memory (Gold, 1986). The diabetic rat offers a model of chronic hyperglycemia which would be a meaningful model to compare with models of transient hyperglycemia following footshock training. Given the present finding of better retention in diabetics, it is conceivable that hyperglycemia does make a contribution to the formation of memory, at least for aversive tasks.

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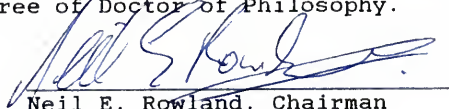
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BIOGRAPHICAL SKETCH

The author was born in Cleveland, Ohio, on August 4, 1943. She completed 2 years of a 3 year nursing program before getting married in 1964. She had 2 children during the next 10 years and began college coursework in 1974, when her youngest child started school. After graduating magna cum laude with a degree in psychology from Cleveland State University, she was accepted in the graduate program at Kent State University, Department of Psychology. She received a Master of Arts degree in general experimental psychology in 1980 and continued graduate work in the Psychology Department, as well as in the Department of Pharmacology of the Northeastern Ohio Universities College of Medicine. In 1982 she moved to the University of Florida where she has been active in research with Dr. Neil Rowland, studying a number of aspects of experimental diabetes in the streptozotocin diabetic rat and working toward the degree of Doctor of Philosophy.

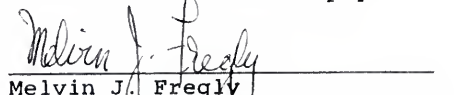
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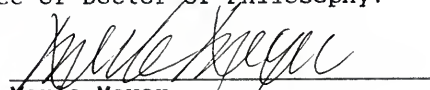
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